A PREPROABRIN:

Genomic cloning and expression of the constituent polypeptides in heterologous systems

by

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DECLARATION

The data contained within this thesis are the results of original research conducted by the author under the supervision of Professor J.M. Lord. All experiments were performed by the author. Where advice or starting material was given this has been acknowledged.

None of the data presented in this thesis have been used in a previous application for a degree.

ABBREVIATIONS

AC	abrin C
AIDS	acquired immunodeficiency syndrome
APA	*Abrus precatorius* agglutinin
b	bases (also kb)
bp	base pairs (also kbp)
Bq	Bequerel (also MBq, TBq)
BSA	bovine serum albumin
C	centigrade
cDNA	complementary DNA
CIAP	calf intestine alkaline phoshatase
Con A	concanavalin A
CTAB	cetyltrimethylammonium bromide
cpm	counts per minute
Da	Dalton (also kDa)
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
(d)dNTP	(di)deoxynucleotide triphosphate (A=adenosine, C=cytidine, G=guanosine, I=inosine, T=thymidine)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EF	elongation factor
ER	endoplasmic reticulum
g	gramme (also mg, µg, ng, pg, fg)
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HIV	Human Immunodeficiency Virus
HPLC	high pressure liquid chromatography
HRV-2	Human Rhinovirus type 2
Ig	immunoglobulin
IPB	immunoprecipitation buffer
IPTG	isopropyl-ß-D-thiogalactopyranoside
l	litre (also ml, µl, nl)

LD_{50}	concentration causing death in 50% of the test group
Ltd.	limited
M	molar (also mM, μM, nM, pM)
MAP	*Mirabilis* antiviral protein
mass/v	mass to volume
MOPS	4-morpholinepropanesulphonic acid
mRNA	messenger RNA
NTP	nucleotide triphosphate (A= adenosine, C= cytidine, G= guanosine, U= uridine)
OD	optical density at wavelength in nm given
OHB	oocyte homogenisation buffer
PAP	pokeweed antiviral protein
PE	*Pseudomonas aeruginosa* exotoxin A
PEG	polyethylene glycol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
plc	public limited company
PMSF	phenylmethylsulphonyl fluoride
PNK	polynucleotide kinase
RCA	*Ricinus communis* agglutinin
RD	ricin D
RE	ricin E
RNA	ribonucleic acid
RNase	ribonuclease
RNAsin	human placental ribonuclease inhibitor
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	salt/sodium citrate
TAE	tris/acetate/EDTA
TBE	tris/borate/EDTA
TBS	tris buffered saline

TBST	tris buffered saline/Tween-20
TCA	trichloroacetic acid
TE	tris/EDTA
TEMED	N,N,N,N'tetramethylenediamine
TEP	tris/EDTA/phosphate
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
ts	temperature sensitive
u	unit
uv	ultra-violet
vsv	vesicular stomatitis virus
v/v	volume to volume
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

Amino acids are represented by both standard one letter and three letter codes. Inorganic salts are represented by standard abbreviations.

SUMMARY

Synthetic oligonucleotides representing all possible sequences of an N-terminal and an internal region of the A-chain of abrin C were used to generate a probe specific for abrin-related sequences using the polymerase chain reaction on *Abrus precatorius* genomic DNA. A lambda phage library constructed from genomic DNA isolated from leaf tissue of *A. precatorius* was screened and positive hybridising clones were characterised by restriction enzyme analysis. The coding regions of unique clones were characterised by DNA sequencing. One clone encodes a preproprotein closely related to abrin C with 83% similarity between the A-chain sequences. Based on the similarity with the ricin toxins and *Ricinus communis* agglutinin, the preproprotein consists of an A-chain of 251 amino acids, preceded by 34 amino acids containing an N-terminal signal peptide, followed by a 14 amino acid linker and a B-chain of 263 amino acids.

The mature A-chain of the preproabrin has been expressed cytoplasmically in *Escherichia coli* and the soluble recombinant protein was produced at levels exceeding 6% of total cell protein. The recombinant A-chain has been purified to homogeneity and its ability to depurinate 28S rRNA in eukaryotic ribosomes has been demonstrated *in vitro*.

The mature B-chain of the preproabrin has been expressed in *Xenopus laevis* oocytes and was found to be unstable and lacking in lectin activity. The non-lectin activity of the protein was confirmed by expression and analysis of the preproabrin in *X. laevis* oocytes.

1. INTRODUCTION

1.1 INTRODUCTION TO TOXIC LECTINS AND RELATED PROTEINS

Many plants serve dual or multiple purposes to man. For many thousands of years the seeds of *Abrus precatorius* and *Ricinus communis* have been known to be highly toxic to man and his cattle. The toxicity is now known to be due to the presence of toxic lectins called abrin and ricin and these proteins are amoung the most poisonous found in nature. The extreme toxicity of abrin and ricin has made these proteins a focus of interest to scientists for many years. The seeds from these plants, or extracts prepared from them, have been used in medicines in many parts of the world for centuries. The criminal fraternity has also realised the potential of such toxic substances.

Abrin occurs in the seed with the related bivalent lectin *A. precatorius* agglutinin (APA). Similarly the seeds of *R. communis*, which contain the toxin ricin, contain the *R. communis* agglutinin (RCA). The agglutins are essentially non-toxic.

Abrin and ricin consist of two dissimilar peptide chains, an A-chain and a B-chain linked by a single disulphide bond, which have different functions. The A-chain catalytically inactivates the 60S subunit of ribosomes in susceptible cells by cleaving a specific adenine residue from the backbone of 28S rRNA (Endo *et al.*, 1987). The B-chain is a lectin and binds to terminal galactose residues on the cell surface leading to cell entry of the holotoxin (Olsnes and Pihl, 1982). There is strong evidence to suggest that a single A-chain molecule within the cytosol can kill a cell (Eiklid *et al.*, 1980).

The agglutinins have very limited cytotoxicty. The A-chain of RCA has the same action as ricin A-chain *in vitro*, but the activity is reduced by several fold. The B-chain of the agglutinins binds to cell surface receptors but there is no progression to productive cell entry and intoxication.

Another type of plant protein, first described from *Phytolacca americana* (Tomlinson *et al.*, 1974), has been found to have very similar properties to the A-chains of abrin and ricin. These proteins, which have now been found in a wide variety of higher plants, lack a cell-binding moeity and are therefore non-cytotoxic. However, *in vitro* they modify ribosomes by the same mechanism of action as abrin and ricin (Stirpe *et al.*, 1986). Modification of the ribosome by cleavage of a single adenine residue from 28S rRNA *in vitro* is not a property exclusive to plant proteins. The Shiga toxin produced by *Shigella dysenteriae* and the Shiga-like toxins of *Escherichia coli* (Calderwood *et al.*, 1987) also modify ribosomes in an identical fashion (Endo *et al.*, 1988a).

Proteins which catalytically inactivate ribosomes by cleavage of the specific adenine residue from the phosphodiester backbone of 28S rRNA are now classified as either type I ribosome-inactivating proteins (single-chain forms) or as type II ribosome-inactivating proteins (heterodimeric forms).

Other bacterial and fungal toxins which include diphtheria toxin found in *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa* exotoxin A (PE), and α-sarcin from *Aspergillus gigantea*, also inhibit protein synthesis but through differing mechanisms of action to the ribosome-inactivating proteins. The A-fragment of diphtheria toxin catalyses the ADP-ribosylation of elongation factor 2 (EF-2) (van Ness *et al.*, 1980). ADP-ribosylated EF-2 cannot translocate the peptidyl-tRNA from the A-site to the P-site although it can still bind in the presence of GTP. Domain III of PE also catalyses the ADP-ribosylation of EF-2 (Hwang *et al.*, 1987). α-sarcin is an endoribonuclease and calalytically inactivates ribosomes by cleavage of a specific phosphodiester bond in 28S, 26S or 23S rRNA (Endo and Wool, 1982).

The ribosome-inactivating proteins have been shown to have anti-viral activity (McGrath *et al.*, 1989) and tumour-suppressive properties (Chang *et al.*, 1985) in addition to

their cytotoxic potential. The extreme cytotoxicity of the toxic lectins, and the *in vitro* activity of the type 1 ribosome-inactivating proteins has made them a focus of research in the development of specific cytotoxic agents, in particular as tumour-specific cytotoxins, by linking them to antibodies (Frankel, 1988). As a consequence of this there is continued interest in determining the details of structure-function relationships, cell entry and substrate specificity.

This thesis describes the cloning and subsequent analysis of an abrin-related protein. The aim of this section is to describe the relevant common knowledge and information known about the toxic lectins at the inception of this project and to provide the context in which the results are discussed.

1.2 BIOSYNTHESIS AND PURIFICATION

Abrin and APA are found amoung the matrix proteins of the protein bodies in the endosperm of *A. precatorius* seeds and constitute up to 30% of the dry weight of the seed (Murray and Vairinhos, 1982). Similarly, ricin and RCA are found in the endosperm protein bodies of *R. communis* seeds (Youle and Hwang, 1976 and Tully and Beevers, 1976). As germination proceeds, the protein body membranes fuse, forming the tonoplast (Ashton, 1976). The contents of the protein bodies remain intact in this vacuole but are later hydrolysed during germination. The toxic lectins are therefore kept out of contact with host ribosomes in the cytoplasm (Tully and Beevers, 1976).

1.2.1 Precursor polypeptides of the toxic lectins

Both ricin and RCA are synthesised as preproprotein precursors which are translated on membrane bound ribosomes in the endosperm cells of maturing seeds (Butterworth and Lord, 1983). The preproproteins consist of a leader peptide, the A-chain, a linker peptide and the B-chain (Roberts *et al.*, 1985 and Lamb *et al.*, 1985). The nascent prolectins are

cotranslationally segregated into the lumen of the endoplasmic reticulum (ER). From the ER they are transported to the protein storage bodies where the linker is cleaved to generate the mature lectin (Harley and Lord, 1985).

1.2.2 Co-translational modifications

The leader sequence presumably contains a signal sequence which mediates translocation of the nascent polypeptide across the ER membrane. The removal of a signal sequence from the precursors of ricin and RCA was demonstrated by immunoprecipitation of the deglycosylated products of *in vitro* translation of mRNA, isolated from ripening castor bean seeds, in the presence or absence of dog pancreatic microsomes (Butterworth and Lord, 1983). The nucleotide sequences of cDNA clones of ricin and RCA (Lamb *et al.*, 1985 and Roberts *et al.*, 1985) and of a genomic clone of ricin (Halling *et al.*, 1985) have been determined and the leader peptide is now known to be 35 amino acids long and is identical in both ricin and RCA. It seems unlikely that the entire leader peptide constitutes the signal peptide. A 35 amino acid leader is longer than most signal peptides (Watson, 1984) and, based on consensus sequences, cleavage after the asparagine residue at position -1 is unlikely. Alternative cleavage sites are proposed after residue -10 (Gly) or residue -20 (Ala), in which case further post translational N-terminal processing must occur (Roberts *et al.*, 1985).

Another co-translational event is core glycosylation (Roberts and Lord, 1981). *N*-glycosylation of a nascent polypeptide occurs in the ER by *en bloc* transfer of the oligosaccharide [$Glc_3Man(GlcNAc)_2$-] from a carrier molecule (Snider and Robbins, 1981 and Hanover and Lennarz, 1981). Pretreatment of intact castor bean endosperm tissue with tunicamycin, inhibited co-translational core glycosylation and glycosylation was not necessary for sequestration of the mature lectin in the protein bodies (Lord, 1985b).

It is believed that disulphide bond formation occurs co-translationally or shortly after release of the propeptide into the ER lumen (Roberts *et al.*, 1987). Ricin and RCA each have a single interchain disulphide bond and four intrachain disulphide bonds in the B-chain.

1.2.3 Post-translational modifications

During transport from the ER to the protein storage bodies there are several post-translational modifications to the *Ricinus* lectins. Cell fractionation of maturing castor bean endosperm, pulse-labelled with [^{3}H]-fucose, has shown that the lectins are transported through the Golgi apparatus during which time the oligosaccharide side chains are modified (Lord, 1985b). This modification includes the addition of fucose, which renders the oligosaccharide moeities partially resistant to endo-N-acetylglucosaminidase H (Lord, 1985b), and the addition of xylose (Kimura *et al.*, 1987 and Kimura *et al.*, 1988b). RCA B-chain contains a fucosylated side chain not present in ricin D B-chain (Lord, 1985a). It is assumed that the lectins are transported in Golgi-derived vesicles to the protein storage bodies where the vesicles fuse to liberate the lectins into the protein storage bodies (Tully and Beevers, 1976).

Once sequestered within the storage bodies there is cleavage of the linker peptide. The linker is identical in both ricin and RCA and is 12 amino acids long and ends with an asparagine residue (Lamb *et al.*, 1985 and Roberts *et al.*, 1985). A partially purified soluble extract from castor bean endosperm protein bodies cleaves the linker from the lectin precursors to generate the mature forms (Harley and Lord, 1985). Plant proproteins commonly undergo endoproteolytic cleavage after an asparagine residue (Lord and Robinson, 1986). It may also be in the protein storage bodies that the final processing of the N-terminus of the A-chain occurs and an

endoprotease with specificity for asparagine may be involved (Roberts *et al.*, 1987).

Roberts and Lord (1981) noted that the molecular mass of the lectins delivered to the protein storage bodies reduces slowly to that of the authentic lectin subunits. This "trimming" can be prevented *in vitro* by the addition of monosaccharides and is not seen *in vivo* for non-glycosylated lectins, synthesised in the presence of tunicamycin, which suggests that this modification involves the removal of sugar residues (Roberts *et al.*, 1987).

1.2.4 Purification

A variety of purification schemes have been devised over the years for purification of the toxic lectins. In the case of type II ribosome-inactivating proteins and the agglutinins, the basis of the purification is affinity chromatography on Sepharose 4B which contains galactose. The toxic lectins bind to the column and contaminating proteins pass through. Ricin is eluted from the column with *N*-acetyl-galactosamine whereas the agglutinin requires galactose for elution (Nicholson and Blaustein, 1972).

The toxic lectins occur in several different forms. Microheterogeneity is seen in seeds of the same source (isoforms) and in seeds of different origin. Certain isoforms differ only in the degree of modification of the oligosaccharide side-chains (Kimura *et al.*, 1988a and Islam and Funatsu, 1988).

Lin *et al.* (1981) isolated four isoforms of abrin termed abrin-a,-b,-c, and -d. Abrin-a is similar to abrin C as described by Wei *et al.* (1974), and to abrin as described by Olsnes and Pihl (1976). Abrin-a binds strongly to Sepharose 4B, is eluted with galactose and is highly toxic. Abrin-b and abrin-c are similar to abrin A (Wei *et al.*, 1974 and Olsnes and Pihl, 1976) and to the abrin A isolated by Herrmann and Behnke (1980) which was found to consist of two isoforms which were

retarded by Sepharose 4B but eluted in the absence of galactose (Herrmann and Behnke, 1981). Abrin-b was noted to be relatively unstable when stored at 4°C compared to the other isoabrins (Lin *et al.*, 1982). Abrin-d has not been described elsewhere. Abrin-d binds to Sepharose 4B and is of reduced toxicity compared to other isoabrins. The abrin which is generally isolated from seed tissue is abrin C (abrin-a) and unless specified abrin refers to abrin C.

A similar situation applies to ricin. The ricin isolated by most laboratories binds strongly to Sepharose 4B and can be eluted with lactose. This isoform is now known as ricin D and unless specified ricin refers to ricin D. Mise *et al.* (1971) described a variant called ricin E which does not bind to Sepharose 4B although binding to cells was inhibited by lactose. The nucleotide sequence of ricin E has been determined and differs from ricin D only in the B-chain (Ladin *et al.*, 1987).

Modeccin, a toxic lectin from the root tissue of *Adenia digitata*, binds to Sepharose 4B (Stirpe *et al.*, 1978) whereas the toxic lectin isolated from the roots of *A. digitata* by Olsnes and Pihl (1982) did not bind to Sepharose 4B but would bind to a column containing desialylated fetuin and was eluted with lactose. Viscumin, the toxic lectin from the leaves of *Viscum album*, occurs as at least two isoforms (Franz *et al.*, 1981). Both viscumin isoforms bind to Sepharose 4B but differ in that one has specificity for galactose and *N*-acetylgalactosamines whereas the other is specific for *N*-acetylgalactosamines alone.

The single-chain type I ribosome-inactivating proteins do not have lectin activity. Several of these proteins have been purified to homogeneity but the individual steps involved in the purification schemes have differed widely. A general procedure has been described for large scale purification of type I ribosome-inactivating proteins which involves the use of a strong cation exchanger (S-Sepharose) and chromatography on CM-Sepharose (Barbieri *et al.*, 1987).

Table 1 summarises the plant-derived ribosome-inactivating proteins which have been described to date. Type I ribosome-inactivating proteins are far more predominant than the toxic lectins.

Table 1 *Ribosome-inactivating proteins from plants*

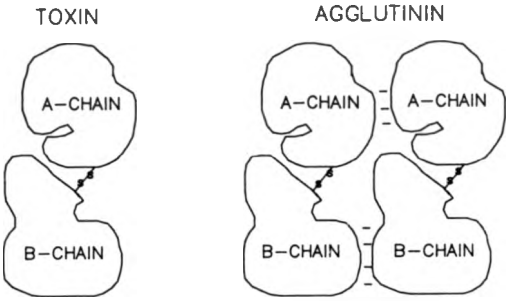
PROTEIN	PLANT SOURCE	TISSUE	REFERENCE
Abrin (A,b,C,d)	*Abrus precatorius*	seed	Lin *et al.*, 1982
A. digitata lectin	*Adenia digitata*	seed	Gasperi-Campani *et al.*, 1980
Asparin (1,2)	*Asparagus officinalis*	seed	Bolognesi *et al.*, 1990
Barley inhibitor	*Hordeum vulgare*	seed	Asano *et al.*, 1986
Bryodin	*Bryonia clioica*	root	Stirpe *et al.*, 1986
Bryodin-R	*Bryonia clioica*	root	Bolognesi *et al.*, 1990
Colocin (1,2)	*Citrullus colocynthis*	seed	Bolognesi *et al.*, 1990
Dianthin (30,32)	*Dianthus caryophyllus*	root & leaf	Reisbig & Bruland, 1983
Dodecandrin	*Phytolacca dodeccandra*	leaf	Ready *et al.*, 1984
E. europaeus lectin	*Euonymus europaeus*	leaf	Gasperi-Campani *et al.*, 1980
Gelonin	*Gelonium multiflorum*	seed	Foa-Tomasi *et al.*, 1982
Luffin (a,b)	*Luffa cylindrica*	seed	Islam *et al.*, 1990; Islam *et al.*, 1991
Lychnin	*Lychrus chalcedonica*	seed	Bolognesi *et al.*, 1990
Mirabilis antiviral protein	*Mirabilis jalapa*	root	Kataoka *et al.*, 1991
Mapalmin	*Manihot palmata*	seed	Bolognesi *et al.*, 1990
Melonin	*Cucumus melo*	seed	Ferreras *et al.*, 1989
-Momorcharin	*Momordica charantia*	seed	Ho *et al.*, 1991
Momorcochin	*Momordica cochinciensis*	root	Yeung *et al.*, 1987b
Momorcochin-S	*Momordica cochinciensis*	seed	Bolognesi *et al.*, 1989
Momordica anti-HIV protein	*Momordica charantia*	fruit & seed	Lee-Huang *et al.*, 1990
Momordin (1,2)	*Momordica charantia*	seed	Yeung *et al.*, 1987a
PAP(I,II,S,R)	*Phytolacca americana*	leaf,seed & root	Irvin, 1975; Irvin *et al.*, 1980; Barbieri *et al.*, 1987a; Bolognesi *et al.*, 1990
P. californicum lectin	*Phoradendron californicum*	leaf	Endo *et al.*, 1989
Ricin (D,E)	*Ricinus communis*	seed	Olsnes & Pihl, 1982
Saporin (1-6)	*Saponaria officionalis*	seed	Lappi *et al.*, 1985
Trikokirin	*Trichosanthes kirilowii*	seed	Cassallas *et al.*,1988
Trichosanthin	*Trichosanthes kirilowii*	root	Yeung *et al.*, 1988
Tritin	*Triticum aestivum*	seed	Reisbig & Bruland, 1983; Roberts & Stewart, 1979
Viscumin	*Viscum album*	leaf	Stirpe *et al.*, 1982
Volkensin	*Adenia volkensin*	root	Barbieri *et al.*, 1984

1.3 STRUCTURE AND PHYSICAL PROPERTIES OF THE TOXIC LECTINS

1.3.1 Primary structure of the toxic lectins

All the type II ribosome-inactivating proteins have molecular masses in the range of 60,000 to 65,000 and consist of two polypeptide chains linked by a single disulphide bond. The A-chain is slightly shorter than the B-chain. The agglutinins consist of two heterodimers, each consisting of an A-chain and a B-chain linked by a single disulphide bond, with the heterodimer held together by weak non-covalent forces (Figure 1).

Figure 1 Schematic structure of the toxic lectins and agglutinins



The primary sequence of ricin has been determined (Funatsu *et al.*, 1979) as has the primary sequence of the A-chain of abrin C (Funatsu *et al.*, 1988). The A-chains are identical at 106 residues and the similarity of 42% suggests a common evolutionary ancestor. The A-chain of abrin C does not contain any glycosylation sites unlike ricin D which has two in the A-chain.

The nucleotide sequences for ricin D, ricin E and RCA have been determined (Lamb *et al.*, 1985, Ladin *et al.*, 1987 and Roberts *et al.*, 1985). The ricin toxins and RCA are remarkably similar in sequence, exhibiting 93% homology at the amino acid level in the A-chains. Ricin D and RCA are 83% homologous at the amino acid level in the B-chains. The N-terminal leader peptide and the linker peptide are identical in all the *Ricinus* toxic lectins.

In total there are only 59 amino acid differences between ricin D and RCA. Of these 59 differences, 45 are caused by a single base change, 10 involve alteration of two bases within a codon and three result from complete codon replacement (Roberts *et al.*, 1985). Of the changes, the majority are not expected to alter the properties of the proteins significantly. The major changes are the presence of two cysteine residues in the A-chain of RCA not present in ricin and an additional glycosylation site within the B-chain of RCA. The effect of the additional cysteines in RCA is not known but they are not expected to be able to form either intrachain or interchain disulphide bonds (personal communication, Dr J.D. Robertus, University of Texas at Austin, Texas, USA). The ricin toxins and RCA have two glycosylation sites within the A-chain at identical positions and two of the glycosylation sites in the B-chain are common to both toxins and RCA. RCA has an additional glycosylation site in the B-chain.

Amino acid sequence analysis of the B-chain of ricin D has indicated that the B-chain sequence arose from a series of gene duplications (Rutenber *et al.*, 1987). The A-chain and B-chain of ricin are not similar.

From the sequence data available on type I ribosome-inactivating proteins, an evolutionary relationship between the A-chains of the toxic lectins and the single chain ribosome-inactivating proteins is postulated (Ready *et al.*, 1984). In addition, ricin A-chain and type I ribosome-inactivating proteins exhibit some similarity to *E. coli* RNase H and to domains of the reverse transcriptase of Moloney murine

leukaemia virus, Rous sarcoma virus and human immunodeficiency virus (Ready *et al.*, 1988). All these proteins bind to and hydrolytically modify nucleic acid. The evolutionary relationship is intriguing because the ribosome-inactivating plant proteins appear to have undergone as much divergence amoung themselves as they have from bacterial and retroviral proteins. Ready and co-workers postulate that the divergence amoung the proteins has reached an equilibrium where the similarity is the minimum needed to maintain function and structural integrity. The ancestral gene may have existed prior to divergence of the common ancestor of plants and eubacteria, or the ribosome-inactivating proteins may have arisen from lateral transfer of a bacterial or viral gene into an ancient angiosperm. Relevant to the latter case there is no evidence to date of ribosome-inactivating proteins in plants below the angiosperm grade although they exist in bacteria (Ready *et al.*, 1988).

The disulphide bond between the A-chain and B-chain of a toxic lectin is not required for toxicity *in vitro* or *in vivo* (Olsnes and Pihl, 1972). In ricin the disulphide bond plays no role in toxicity except to maintain the association between the two chains at low concentrations (Lewis and Youle, 1986). Olsnes and Pihl (1982) reported that irreversible covalent cross-linking of abrin with dimethyl suberimidole resulted in non-cytotoxicity. Similar results were obtained with the cross-linking of ricin with N,N'-o-phenylenedimaleimdate (Oda and Funatsu, 1979). It has been demonstrated that the disulphide bond is more readily reduced in the presence of SDS (Olsnes and Pihl, 1982) which might indicate that a conformational change which occurs during binding to the cell surface, or during transport to the cytosol, would facilitate reduction of the interchain disulphide bond.

The amount of carbohydrate present in ricin and abrin vary significantly in the published data. The toxic lectins and the agglutinins all contain mannose and glucosamine (Olsnes and

Pihl, 1982) and abrin, ricin and modeccin bind to concanavalin A which suggests that the mannose residues are exposed.

The net charge of the toxic lectins has been determined in several laboratories. The A-chains of type II ribosome-inactivating proteins and the type I ribosome-inactivating proteins typically have a neutral or basic pI. Abrin C A-chain is apparently unusual in having an acidic pI.

The overall charge on the holotoxin was demonstrated to have little effect on toxicity *in vitro* using combinations between the A-chain of ricin and the B-chain of abrin, and vice versa, to generate molecules with either acidic or neutral overall charge respectively (Olsnes *et al.*, 1974a).

1.3.2 3-dimensional structure

Montford *et al.* (1987) determined the three-dimensional structure of ricin at 2.8Å by X-ray crystallography. Ricin A-chain is a globular protein with extensive secondary structure. A prominant cleft at the interface of the A-chain with the B-chain is postulated as the active site of the A-chain. The B-chain exists as two globular domains which have similar topology. Each B-chain domain hydrogen bonds to a molecule of galactose which lies in a shallow cleft. The interaction between the A-chain and B-chain shows some hydrophobic interactions in which proline and phenylalanine play an important role.

1.3.3 Immunocytochemistry and antibody formation

The toxic lectins abrin, ricin and modeccin are immunologically distinct (Godal *et al.*, 1981 and Olsnes *et al.*, 1978). There is some cross reactivity in some sera between ricin and abrin which, as determined by radioimmunoassay, is limited to approximately 2% compared to binding with homologous toxin (Godal *et al.*, 1981). There is no cross reactivity between sera against the isolated A-chains and B-chains

(Pappenheimer *et al.*, 1974). Ricin will induce antibodies that cross react with RCA and *vice versa* (Pappenheimer *et al.*, 1974). Antibodies against abrin however do not cross react with APA which suggests that the *Abrus* lectins differ more than the *Ricinus* lectins (Olsnes and Pihl, 1982).

Godal *et al.* (1983) used an enzyme-linked immunosorbent assay to study antibody formation in mice and humans against abrin and ricin. Abrin was found to be strongly immunosuppressive and more efficient than cyclophosphamide in inhibiting anti-human serum albumin formation in mice. In humans treated with therapeutic doses of abrin, the patients remained free of anti-abrin antibodies for approximately 10 weeks compared to about 7 weeks for the production of anti-ricin antibodies in patients given eqivalent doses of ricin.

1.3.4 Resistance to physical treatment

Holotoxins are stable for long periods of time in the frozen state and toxicity is altered little by repeated freeze-thawing cycles. Abrin and ricin are stable for several months at 4°C in the presence of 0.1M galactose. Ricin is marginally more resistant to heat than abrin, the latter being inactivated by heating to 60°C for 1 hour whereas ricin retains some activity (Olsnes and Pihl, 1982).

The isolated chains are less stable and require 10% glycerol to be present for freezing without loss of activity. The isolated B-chains are significantly less stable than the isolated A-chains (Wawrzynczak and Thorpe, 1986). The isolated chains can be stored at 4°C for several months in phosphate buffered saline, with 0.1M galactose in the the case of the B-chains, without significant drop in activity although a 10-100 fold drop in activity of recombinant ricin A-chain stored at 4°C has been noted (personal communication, Dr M.R. Hartley, University of Warwick, UK).

1.3.5 Chemical modification

In the development of immunotoxins the effect of chemical modifications of the toxic lectins continues to be of interest. The holotoxins of abrin and ricin are resistant to treatment with a wide variety of proteolytic enzymes (Balint, 1974). The denatured holotoxins and the isolated chains are more susceptible to proteases (Leignier *et al.*, 1976 and Yoshitake *et al.*, 1979).

Funatsu *et al.* (1977) found that nagarase released at least 18 amino acids from the N-terminus of ricin A-chain but did not affect the B-chain. Loss of the N-terminus which includes one of the oligosaccharide side-chains did not alter toxicity. There have been several reports of peptides from limited hydrolysis of ricin A-chain which were as active or more active than native ricin A-chain on cell-free systems (Olsnes *et al.*, 1982) but the peptides have not been well characterised.

Ricin and abrin are totally inactivated by treatment with oxidising agents (Balint, 1974) and the B-chain of ricin is highly susceptible to chloramine-T treatment (Sandvig *et al.*, 1976). Methylation of abrin and ricin did not significantly alter toxicity *in vitro*, but the ability of the B-chain to bind saccharides was abolished. Similar results were obtained with acetylation of abrin and ricin (Sandvig *et al.*, 1978b). Houston (1983) showed that chemical modification of ricin with 4-diazophenyl-B-D-galactopyranaside inactivated the B-chain but did not alter toxicity *in vitro*.

APA retained binding activity after modification of lysine, tyrosine, arginine, histidine, glutamate and aspartate residues by a variety of methods (Patanjali *et al.*, 1984). Modification of tryptophan residues with N-bromosuccinimide led to complete loss of carbohydrate binding by APA (Patanjali *et al.*, 1987). Modification of the tryptophan residues in ricin and abrin with 2-hydroxyl-5-nitrobenzyl bromide did not significantly alter toxicity *in vitro* (Olsnes and Pihl, 1982).

1.4 TOXICITY OF THE TOXIC LECTINS

1.4.1 Animals and man

The type II ribosome-inactivating proteins are highly toxic to mammals, particularly when administered parenterally. Animals show different sensitivities to the toxic lectins with horses being the most sensitive of those studied to date (Balint, 1974). The data from different laboratories on the toxicity of the toxic lectins are difficult to compare because the purity, doses, administration routes and scoring methods differ. Olsnes and Pihl (1982) found that the LD_{50} dose of abrin after intravenous injection in mice was 0.7µg/kg, in rabbits was 50ng/kg and in dogs was 1.25ng/kg.

After administration of toxin a lag period is seen during which time there are no symptoms. There follows a period during which weakness, weight loss, diarrhoea and fever are the typical symptoms. The white blood cell count rises transiently to up to 5 fold of the norm. Survival time is dose dependent. Animals given non-lethal doses of abrin or ricin recover completely (Olsnes and Pihl, 1982).

In rats given lethal doses of ricin the liver, spleen and pancreas become severely necrotic and the reticulo-endothelial cells seem particularly sensitive. Protein synthesis is most severely inhibited in the pancreas and spleen. Non-parenchymal cells in the liver take up ricin and abrin more rapidly than parenchymal cells and protein synthesis is inhibited *in vitro* to a far greater extent in non-parenchymal cells than in parenchymal cells (Skilleter *et al.*, 1981).

1.4.2 Cultured cells

There is no correlation between toxicity in animals and cultured cells. Ricin E is as toxic to mice as ricin D but less toxic to cultured cells (Mise *et al.*, 1977). Different cell lines exhibit a wide degree of sensitivity to the toxic

lectins. The different IC_{50} found for cultured cell lines reflects differences in the number of cell surface receptors, efficiency of transport mechanisms, rate of degradation of internalised toxin, rate of redistribution of the contents of endocytosed vesicles to the cell surface and the inherent susceptibility of the ribosomes to the action of the toxin. Abrin, ricin and modeccin can kill cells in culture at doses as low as 1ng/ml.

After addition of abrin or ricin to cells there is always a lag time of 20-30 minutes even in the presence of an excess of toxin (Olsnes and Pihl, 1982). The first demonstrable effect is inhibition of protein synthesis, followed by a decrease in DNA synthesis and then RNA synthesis (Bennet *et al.*, 1982 and Witten *et al.*, 1982).

1.5 MECHANISMS OF ACTION OF THE A-CHAIN

1.5.1 Inactivation of ribosomes

Endo and Tsurugi (1986) noted that the 28S rRNA from ricin treated ribosomes migrated slightly slower than that from untreated ribosomes and that the modification to the 28S rRNA did not involve phosphorolysis. The modification site was found to lie within a 553 nucleotide fragment that is normally generated by the contaminating nucleases. This fragment was sequenced and found to be derived from the 3'-terminal of 28S rRNA. By end-labelling the rRNA from ricin treated ribosomes followed by partial digestion with RNases it was shown that the bases corresponding to G-4323 and A-4324 of 28S rRNA had been modified (Endo *et al.*, 1987).

Partial alkaline hydrolysis indicated that the lability of the phosphodiester bonds adjacent to A-4324 was increased (Endo *et al.*, 1987). This result implied that the C-1 carbon of the ribose of the modified residue had been altered to an aldehyde by cleavage of the *N*-glycosidic bond. Treatment of the modified rRNA with aniline at acidic pH resulted in scission of the

phosphodiester bonds adjacent to A-4324 and indicated that A-4324 was the base modified by ricin and related toxins (Endo *et al.*, 1987). Direct evidence that ricin A-chain inactivates ribosomes by cleaving the *N*-glycosidic bond of A-4324 of 28S rRNA in a hydrolytic fashion was obtained by Endo and Tsurugi (1987) who demonstrated the release of 1 mole of adenine from 1 mole of ribosomes by ricin A-chain.

Ricin was also shown to modify naked 28S rRNA but the rate and amount of modification was reduced (Endo *et al.*, 1987). The ricin to substrate ratio for modification to occur was increased by 10,000 fold. Denatured naked 28S rRNA was not modified nor was the isolated 553 nucleotide fragment (Endo *et al.*, 1987). This suggested that some secondary structure was important for action of ricin. Endo *et al.* (1988a) showed that ricin retained activity when tested on a 35-mer that mimics the region modified in 28S rRNA. The oligoribonucleotide forms a stem loop structure with the base modified by ricin lying on the exposed loop. The concentration of ricin required to modify the 35-mer was 10,000 times greater than that required to modify the 28S rRNA in intact ribosomes which indicates that the higher order structure of this loop is different in ribosomes than it is in naked rRNA.

The elucidation of the site and mechanism of action of ricin and related toxins led to the development of a simple assay for detecting the activity of ribosome-inactivating proteins: ribosomes are incubated with the ribosome-inactivating protein, the rRNA is extracted and treated with aniline to release a fragment of rRNA from any modified 28S rRNA, and the released fragment is visualised by electrophoresis and staining with ethidium bromide. Using this assay the *N*-glycosidase activity of all the ribosome-inactivating proteins known to date has been confirmed using rabbit liver ribosomes (Endo *et al.*, 1988b, Endo *et al.*, 1988c, Endo *et al.*, 1988d, Endo *et al.*, 1988e and Stirpe *et al.*, 1988).

Aspergillus giganteus produces a small basic protein called α-sarcin which inactivates ribosomes by hydrolysing the phosphodiester bond on the 3' side of G-4325 in rat liver ribosomes (Endo and Wool, 1982). The cleavage site lies within a purine-rich domain of 14 residues which are highly conserved in all organisms, commonly referred to as the α-sarcin domain, and forms a stem loop structure (Chan *et al.*, 1983). Table 2 lists the α-sarcin domain from a variety of ribosomes. It is identical in cytoplasmic rRNAs of mammals, *Xenopus laevis*, and *Saccharomyces cerevisiae* and is largely conserved in organelle rRNAs. α-sarcin modifies the ribosomes of all organisms that have been tested to date, by cleavage of the single phosphodiester bond. The finding that cleavage of a single phosphodiester bond, or removal of an adenine residue from the phosphate backbone, of the α-sarcin domain indicates that this domain is crucial for function. Ribosomes do not need intact rRNA *per se* as ribosomes can survive despite treatment with nucleases which introduce many nicks (Cahn *et al.*, 1970).

Table 2 *Comparison of the sequence of the α-sarcin domains* Residues which differ from the rat liver ribosome sequence are shown in bold. The arrow indicates the site of cleavage by α-sarcin and the asterisk indicates the base modified by ricin

RIBOSOMES	SEQUENCE	REFERENCE
Rat liver	5'-AGUACGAGAGGAAC-3' *↑	Endo and Wool, 1982
S. cerevisiae	AGUACGAGAGGAAC	Endo and Wool, 1982
E. coli	AGUACGAGAGGACC	Edwards & Kossel, 1981
Z. mays chloroplast	AGUACGAGAGGACC	Edwards & Kossel, 1981
S. cerevisiae mitochondria	AGUACGC**A**AGGACC	Sor & Fukuhara, 1983
X. laevis oocyte	AGUACGAGAGGAAC	Saxena & Ackerman, 1990

1.5.2 Steps inhibited in protein synthesis

The A-chain of the type II ribosome-inactivating proteins and the single-chain ribosome-inactivating proteins alter the conformation of the ribosome. Terao *et al.* (1988) used a double-labelling technique in which ribosomes were incubated with ricin, or α-sarcin, and then treated with [^{3}H]-labelled or [^{14}C]-labelled N-ethylmaleimide to investigate conformational alterations in the ribosome. Ricin specifically reduced labelling of protein L14 in 80S ribosomes and α-sarcin reduced labelling of proteins L3 and L4. The proteins L3, L4 and L14 are all neighbours. Pretreatment of 60S ribosomal subunits with ricin facilitated the release of a 5S RNA-L5 protein complex by brief heating (Paleologue *et al.*, 1986). L5 is a neighbour of L3 and all the proteins L3, L4, L5 and L14 are located at the boundary between the 40S and 60S subunits and interact with mRNA (Takahashi and Ogata, 1985). L3 and L5 are located at the P-site and L4 and L5 can be cross linked with EF-2. The conformation change in the ribosome may be associated with, or the cause of, the inhibition of protein synthesis by ribosome-inactivating proteins.

The particular steps in protein synthesis which are affected by ribosome-inactivating proteins have not been fully elucidated. There is some evidence that initiation is inhibited. Fernandez-Puentes and Vasques (1977) used reconstitution experiments between 40S and 60S ribosomal subunits pretreated with ricin to show that the 60S subunit is the only component of the translation apparatus affected. Skorve *et al.* (1977) showed that abrin A-chain caused a strong reduction in the amount of labelled mRNA in the 80S subunit whereas the binding of labelled mRNA or methionine to the 40S subunit was unaffected. They concluded that the formation of the 80S subunit from the 60S and 40S subunits was inhibited by the action of abrin A-chain.

It is now generally accepted that ribosome-inactivating proteins affect the elongation cycle of protein synthesis. EF-2

protects ribosomes from the action of ricin and this protective effect is enhanced by the presence of GTP or non-hydrolysable GTP analogues. The protection was reduced in the presence of Phe-tRNA, EF-1 or in high magnesium ion concentrations (Fernandez-Puentes *et al.*, 1976). The protection against ricin by aminoacyl-tRNAs suggests that toxins cannot inactivate ribosomes when the acceptor site is occupied. Olsnes and Pihl (1982) postulate that in ricin treated ribosomes functional groups are located more distantly to each other than usual, and in the presence of high magnesium ion concentrations, these groups resume the normal position in the more compact ribosome structure that is aquired. Protection by EF-2 to the action of ricin suggests that the region in the 28S rRNA modified by the ribosome-inactivating proteins is identical or close to the EF-2 binding site.

Ricin inhibits the binding of limiting concentrations of EF-2 to ribosomes (Nilsson and Nygard, 1986 and Montanaro *et al.*, 1975) and affects the activities of the EF-1 and EF-2-dependent GTPases (Sperti *et al.*, 1975, Fernandez-Puentes and Vasquez, 1977, Benson *et al.*, 1975 and Benson, 1977). The peptidyl-transferase component is unaffected by the action of ricin (Gessner and Irvin, 1980).

Olsnes *et al.* (1975a) estimated that ricin and abrin can inactivate up to 1500 ribosomes per minute. In a cell free system elongation was stopped when only one or a few ribosomes per polysome were inactivated (Fodstadt and Olsnes, 1977). Youle and Neville (1979) found that in polysomes pretreated with ricin, EF-2 is tightly bound to the polysomes rendering the ribosomes resistant to ADP-ribosylation by diphtheria toxin and from modification by the addition of ricin A-chain. The majority of the ribosomes when released from the polysome, by denaturing agents in the presence of anti-ricin antibodies, retained protein synthesising activity (Fernandez-Puentes *et al.*, 1976).

Until recently little was known of the role of individual ribosomal domains or ribosomal components. For many years the

ribosomal proteins were considered to be the active components of the ribosome and the rRNAs provided the scaffolding on which the partial reactions of protein synthesis occured. The view today is that the rRNAs are responsible for the biochemistry of protein synthesis, and the ribosomal proteins facilitate the folding and maintenance of the optimal configuration of the rRNA.

1.5.3 Liberation of the A-chain

Both *in vivo* and *in vitro*, reduction of the disulphide bond between the two chains of the holotoxins is an absolute requirment for A-chain activity (Olsnes and Pihl, 1982). The single-chain toxins are unaffected by reducing agents. Rabbit reticulocyte lysate contains sufficient reducing agent to rapidly break the interchain disulphide bond and glutathione probably accounts for most of this activity (Barbieri *et al.*, 1982b).

The site within the cell at which the interchain disulphide bond is broken remains unknown. Endocytic vesicles can have a pH as low as 5.5 which would dramatically slow the rate of reduction of disulphide bonds (Mellman *et al.*, 1986). Compartments within the Golgi apparatus have a higher pH and are likely to contain disulphide exchange enzymes (Youle and Colombatti, 1986). Reduced ricin and abrin is more toxic to cultured cells than the native toxins therefore the reduced toxins may not require transport to the compartment where reduction of the disulphide bond occurs (Olsnes and Pihl, 1972 and Lewis and Youle, 1986). The interchain disulphide bond is not required for toxicity but initiation of entry of the A-chain into the cytosol is dependent upon the B-chain either covalently linked or in an associated form (Lewis and Youle, 1986). Evidence exists that there is a conformational change in the subunits of ricin on reduction of the disulphide bond (Olsnes *et al.*, 1975b).

1.6 FUNCTION OF THE B-CHAIN

1.6.1 Binding to sugars and to complex carbohydrates

Binding of the B-chains of the toxic lectins to a variety of sugars has been carried out by several different techniques. The association constants of abrin and ricin for lactose have been determined from equilibrium dialysis (Olsnes and Pihl, 1972), temperature jump relaxation studies (Podder *et al.*, 1978) and microcalorimetric methods (Zentz *et al.*, 1977) and was of the order $Ka=10^4\ M^{-1}$. The affinity for asialofetuin of the toxic lectins is several orders of magnitude higher and is of the order $Ka= 1\text{-}5 \times 10^8\ M^{-1}$ (Sandvig *et al.*, 1978c). Typically only one lactose molecule per B-chain was found although evidence for two lactose binding sites was obtained (Villafranca and Robertus, 1981 and Houston and Dooley, 1982). From the X-ray crystallographic data, ricin binds 2 lactose molecules per B-chain (Montford *et al.*, 1987) and the mode of binding at the two sites is similar.

Hatakeyama *et al.* (1986) demonstrated that the two lactose binding sites of ricin D have different specificities. The low affinity site, which lies in the first globular domain of the B-chain, binds to galactopyranosides but lacks ability to bind *N*-acetylgalactosamines. The high affinity site lies in the second globular domain of the B-chain and can bind to both galactopyranosides and *N*-acetylgalactosamines.

The agglutinins exhibit the binding properties of the low affinity site of ricin D in that they lack binding ability to *N*-acetylgalactosamines (Nicholson and Blaustein, 1972) and there is evidence that RCA only binds one molecule of lactose per B-chain (Podder *et al.*, 1978 and Houston and Dooley, 1982).

Baenzinger and Fiete (1979) studied the specificity of binding of ricin and RCA to branched asparagine-linked oligosaccharides. They determined that β-1,4-linked galactose residues were primarily responsible for binding. The *Abrus* and *Ricinus* lectins bind to the same kind of oligosaccharides but

the strength of binding differs (Ishiguro *et al.*, 1964 and Green *et al.*, 1987).

1.6.2 Binding to cell surfaces

Ricin and abrin bind to a large number of cell surface glycoproteins and glycolipids which contain terminal galactose residues. The number of sites per cell varies between cell types and is typically from between 10^6 and 10^8 toxin molecules per cell (Sandvig *et al.*, 1976 and Sandvig *et al.*, 1978a). In the case of ricin it seems likely that only the high affinity site strongly binds to cells since modification of the tyrosine residue at the high affinity site dramatically reduces cell binding (Sandvig *et al.*, 1978b).

Ricin and abrin are also bound by mannose receptors through recognition of its mannosylated *N*-linked oligosaccharides. The intoxication of macrophages in the presence of lactose indicated that cell binding via the mannose receptors led to efficient cell killing (Simmons *et al.*, 1986 and Tsuzuki and Wu, 1982). Skilleter *et al.* (1986) demonstrated that uptake by non-parenchymal cells is principally through mannose recognition. Deglycosylation of the toxin prevents entry by this pathway (Foxwell *et al.*, 1984)

To study the binding of the lectins to cell surfaces immunocytochemical detection and ligand conjugates have been used. The lectin is visualised by probing with ferritin or colloidal gold, or by enzymatic means with peroxidase. Monovalent and polyvalent conjugates of ricin and peroxidase bind evenly to the cell surface at $4^{o}C$ (van Deurs *et al.*, 1987a). Washing of cells, after ricin binding, at $4^{o}C$ followed by further incubation at $37^{o}C$ does not alter the even distribution of the ricin conjugates. These results indicate that the binding sites are initially evenly distributed over the cell surface and internalisation occurs relatively slowly. It is possible that some binding sites are not internalised at all. Similar experiments with transferrin showed that the

transferrin receptor is associated with coated pits in the absence of any ligand (Sandvig *et al.*, 1987).

Ricin-gold conjugates are evenly distributed on the cell surface at 4°C but are rapidly endocytosed at 37°C and the conjugate which remains at the cell surface forms patches (van Deurs *et al.*, 1985). It seems likely that the polyvalent gold-ricin conjugates induce receptor redistribution and aggregation at 37°C. Cell surface associated ricin or monovalent ricin-peroxidase conjugates can be washed off with lactose whereas polyvalent or ricin-gold conjugates remain associated with the cell indicating the higher binding specificity of the polyvalent conjugates (van Deurs *et al.*, 1986 and van Deurs *et al.*, 1985).

Some ricin remains at the cell surface even after internalisation at 37°C, at least until the cell becomes intoxicated. The overall rate of internalisation is slow, some binding sites are not internalised and there is rapid recycling of some toxin back to the cell surface (Sandvig *et al.*, 1976).

1.6.3 A role in translocation

Ricin A-chain has been extensively used in the generation of immunotoxins where the A-chain has been linked to a tumour-specific antibody. In several instances the immunotoxin had limited cytotoxicity and in some cases was non-cytotoxic. McIntosh *et al.*(1983) found that a non-cytotoxic antibody-ricin A-chain conjugate could be converted into a potent specific cytotoxic agent by the addition of ricin B-chain as a secondary agent. The cytotoxicity of the combined reagents was not altered by the presence of lactose in the medium which strongly argues against the B-chain inducing a signal to internalise the conjugates and suggests some association between the ricin A-chain and B-chain.

Houston (1983) has shown that ricin A-chain can reassociate with B-chain that has already bound to cell surfaces. A synergistic effect of ricin B-chain has been

demonstrated in a different system where ricin A-chain and ricin B-chain were separately linked to an anti-IgG antibody (Vitetta *et al.*, 1983). In this experiment the specificity of the antibody was found to be important in that a B-chain linked to an antibody of irrelevant specificity did not synergise the effect of the A-chain on Daudi cells (an IgG bearing cell line). This suggested that either both immunotoxins were binding to the same receptor and being endocytosed into the same compartment so that free A-chain associated with B-chain is formed in the endosome, or that the A-chain and B-chain reach the same compartment via separate endocytic routes. In either case the B-chain is enhancing translocation of the A-chain into the cytosol, a function distinct from cell surface binding.

Fulton *et al.* (1986) demonstrated the potentiation by B-chain using a "piggyback" immunotoxin. In this system an IgG, linked to ricin A-chain, against a surface immunoglobulin of Daudi cells are bound to the cell surface followed by the addition of ricin B-chain linked to an IgG specific for the antibody portion of the A-chain immunotoxin

It has been suggested that the galactose binding domains are not involved in the potentiating effect of the B-chain. In immunotoxins where the B-chain has been blocked by the linkage to antibody (Thorpe *et al.*, 1984), or by chemical modification (Thorpe *et al.*, 1985), sufficient to prevent galactose binding by the B-chain, the B-chain moeity was still capable of potentiation. Vitetta (1986) postulates that either the galactose-binding domain is distinct from the 'potentiating domain' or that the 'potentiating domain' is less sensitive to changes in tertiary structure. It may also be a consequence of the unblocking of the galactose-binding sites of the B-chain during or after internalisation due to a conformational change, or in the case of chemically modified B-chain there is not complete abolishment of binding (Vitetta, 1986) and the small amount of binding may be sufficient for translocating the A-chain. It has therefore not been determined if the potentiating

effect of the B-chain is due entirely to galactose binding within the cell or if a second distinct functional domain exists.

1.7 INTERNALISATION OF THE TOXINS

1.7.1 Rate of uptake

Kinetic experiments suggest that only one molecule of abrin or ricin in the cytosol can kill a cell (Euklid *et al.*, 1980). It is clear that the process through which the lectin, or the A-chain, reaches the cytosol is inefficient since several thousand molecules of the lectin must be bound to the cell surface to ensure intoxication (Tsuzuki and Wu, 1982).

After the addition of toxic lectins to cells there is always a lag-time which is strongly temperature dependent (Hudson and Neville, 1987). This lag-time cannot be reduced to less than about 20 minutes. During this lag-time initially both anti-A-chain and anti-B-chain antibodies afford protection followed by a period in which only anti-A-chain antibodies are effective (Houston, 1982). During the major part of the lag-time antibodies have no major effect. The initial stage of entry therefore involves binding of the B-chain to the cell surface followed by penetration of the toxin through the cell membrane.

By removing the non-endocytosed toxin from the cell surface it is estimated that approximately 15% of total cell associated ricin is endocytosed within 10 minutes (Sandvig *et al.*, 1978a and Sandvig and Olsnes, 1982a). A lag time is never seen when ricin is delivered directly to the cytosol, by fusion of erythrocyte ghosts or viral envelopes loaded with ricin A-chain with the cell membrane (Sargiacoma *et al.*, 1983 and Foxwell *et al.*, 1984). The major part of the lag-time therfore involves the transport of the A-chain to the intracellular target and the rate limiting step is transport to the cytosol (Hudson and Neville, 1987).

The internalisation of many physiological liganda is far more rapid than that of ricin and abrin (Ciechanover *et al.*, 1983 and Bleil and Bretscher, 1982). As much as 60% of transferrin is internalised within 10 minutes by Vero cells (van Deurs *et al.*, 1987b). It seems likely that ricin and related toxins bind to a wide variety of cell surface molecules of which many may by essentially stationary molecules or are internalised only very slowly.

1.7.2 Role of endocytosis

Evidence has accumulated that the toxins abrin and ricin are translocated to the cytosol from intracellular structures. The involvement of endocytosis in the penetration of a toxin was first demonstrated with diphtheria toxin (Sandvig and Olsnes, 1980). Diphtheria toxin requires transport to an acidic compartment for translocation and only the intracellular vesicles or vacuoles have the required low pH of less than 5.3.

Abrin and ricin do not require low pH and under conditions where the acidification of intracellular vesicles is inhibited the cells are sensitised to the toxins (Sandvig *et al.*, 1982a and Sandvig *et al.*, 1982b). The evidence to support transport in endocytotic vesicles comes from studies in which endocytosis occurs but translocation is prevented. Cells incubated in the absence of calcium ions take up ricin but translocation is prevented (Sandvig and Olsnes, 1982a). If the cells are washed with lactose and treated with anti-ricin antibodies, then incubated overnight in the presence of calcium, the cells are intoxicated to the same extent as cells treated with ricin in the presence of calcium. These results indicate that endocytosed ricin was capable of intoxicating cells without returning to the cell surface and that translocation from endocytic vesicles is a major entry pathway (Sandvig and Olsnes, 1982a).

An as yet unresolved question is how abrin and related toxins are endocytosed. There is considerable evidence that

many molecules are endocytosed via the coated pit pathway which include transferrin and viruses (Marsh, 1984 and Pearse and Bretscher, 1981). The presence of clathrin as a marker in coated pits has allowed the vesicles derived from coated pits to be determined. It is clear from several studies that at least some ricin is endocytosed via this route (van Deurs *et al.*, 1985 and Sandvig *et al.*, 1987), but it remains to be determined if this is the productive entry route and does not exclude the possibility of alternative mechanisms.

Smooth or uncoated areas on the cell surface are also likely to be involved in endocytosis and there is increasing evidence that ricin may be endocytosed through a non-clathrin coated endocytic pathway. Moya *et al.* (1985) and Madshus *et al.* (1987) showed that potassium depletion in combination with brief hypotonic shock led to the loss of virtually all coated pits on the suface of Hep-2 cells. Under these conditions the uptake of ricin was virtually unchanged as was the effect of human rhinovirus type 2 (HRV-2), whereas the uptake of transferrin was completely inhibited. These results indicated that ricin, and HRV-2 which requires translocation to an acidified vesicle, are taken up by a non-clathrin endocytic pathway.

In support of this evidence, Sandvig *et al.*(1987) showed that acidification of the cytosol of various cell lines by different methods inhibited translocation of transferrin but only mildly reduced the uptake of ricin. The number and distribution of transferrin-receptors was unaltered in the acidified cells and it was postulated that acidification prevents the formation of vesicles derived from clathrin-coated pits thereby preventing endocytosis of transferrin-ligand complexes. Tetanus toxin and cholera toxin are apparently internalised through smooth pits (Montesano *et al.* 1982) and bind to glycolipids for which ricin and abrin also have binding affinity. It seems likely that ricin can bind to surface receptors not involved in coated pits and be internalised.

In summary it is clear that some ricin is endocytosed via the clathrin-coated pit pathway and therefore imitates physiologically important ligands such as transferrin which binds to glycoprotein receptors. There is also evidence to support endocytosis of ricin through an alternative pathway but lack of a specific marker for cell surface molecules which are excluded from coated pits makes characterisation of this alternative pathway difficult.

1.7.3 Intracellular trafficking of ricin

The endosomal system

Ricin has been detected in endosomes by pre-embedding immunoperoxidase cytochemistry (van Deurs *et al.*, 1986) and postembedding immunogold cytochemistry (van Deurs *et al.*, 1985) and both ricin-peroxidase and ricin-gold complexes have been traced to endosomes within a few minutes of exposure to the cells (van Deurs *et al.*, 1987a). Many ligands and receptors dissociate under the low pH conditions to allow further sorting and cycling of the receptor back to the cell surface. Ricin remains attached to its receptor in the endosome and only about 20% of the ricin bound at pH 7 was released at pH 5 (van Deurs *et al.*, 1987b). The majority of ricin which is recycled to the cell surface remains intact.

The valency of the ligand can affect sorting of molecules in the endosomal system. Polyvalent ricin conjugates are delivered to lysosomes and the trafficking of transferrin-gold conjugates is altered (van Deurs *et al.*, 1986). Monovalent and polyvalent ricin-peroxidase conjugates are detected in endosomes but only the monovalent conjugate reaches the Golgi network (van Deurs *et al.*, 1985 and van Deurs *et al.*, 1986).

The concept of a distinct population of endosomes with a vesicular shuttle to the lysosomes (Helenius *et al.*, 1983) has now been replaced with the concept of a maturation from early endocytic compartments (endosomal) to late endocytic compartments (lysosomal). It is also becoming evident that the

endosomal system may consist of an interconnected system rather than discrete vacuolar or tubular structures. Ricin which reaches late endocytic vesicles is slowly degraded, either due to slow delivery or to resistance to proteolytic enzymes. After 60 minutes up to 35% of the internalised ricin is found in lysosome-like vesicles (van Deurs *et al.*, 1986).

The trans-Golgi network

The glycosylated 57kDa membrane protein of vesicular stomatitis virus (VSV) has been used as a marker of the secretory-Golgi pathway of cells exposed to ricin. In cells infected with VSV, the G protein is synthesised and within 30 minutes is transported from the ER, via the Golgi network, to the cell surface. A temperature sensitive mutant of VSV, ts 045, leads to synthesis of the G-protein at the non-permissive temperature of 39.5^{o}C but it is retained within the ER. On shifting to 20^{o}C, the G protein leaves the ER but accumulates in the Golgi in large amounts, particularly in the trans-Golgi network and virtually no G protein reaches the cell surface. At 31^{o}C, the permissive temperature, the G protein is rapidly transported via the Golgi-network to the cell surface (van Deurs *et al.*, 1987b).

Cells infected with VSV ts 045 were incubated with ricin for 60 minutes at 39.5^{o}C after which time ricin was found in both early and late endocytic vesicles and in some Golgi-associated structures and the G protein remained in the ER. On shifting to 20^{o}C the G protein moved into the Golgi-complex but further transport of ricin to Golgi-associated structures is prevented. Using double-labelling of ultrathin cryosections of the infected cells incubated with ricin about 4% of the total internalised ricin was found to be colocalised with the G-protein in the Golgi-network of which about 75% was in the trans-Golgi network (van Deurs *et al.*, 1987b).

Ricin has also been noted in Golgi compartments using preembedded immunoperoxidase cytochemistry (van Deurs *et al.*, 1986). Conflicting results on the delivery of ricin to the

Golgi-network have been obtained using ricin-conjugates. Monovalent ricin-conjugates are delivered to the Golgi-associated compartments whereas polyvalent conjugates are rarely seen there. Observations by several workers indicate that the sorting of the polyvalent conjugates is altered during the stages prior to reaching Golgi-associated compartments.

Translocation to the cytosol

Increasing evidence now points to the trans-Golgi network as the compartment from which ricin translocates to the cytosol. Toxins which translocate from endocytic compartments such as diphtheria toxin A-chain, rapidly inhibit protein synthesis and incubation of cells at 18°C has little effect on toxicity, whereas toxicity is prevented by increasing the pH in the endosomal compartments (Sandvig and Olsnes, 1980 and Sandvig and Olsnes, 1981). The long lag-time before inhibition of protein synthesis by the toxic lectins, the sensitisation of cells to ricin and abrin on increase in pH of endosomes and inhibition of the action of ricin at 18°C which prevents transport to the Golgi-associated compartments, all indicate that translocation occurs from the Golgi-network (Sandvig and Olsnes, 1982a and Sandvig and Olsnes, 1982b).

Monensin, which sensitises cells to ricin and abrin, alters the morphology of the Golgi-complex (Tartakoff, 1983). Cyclohexamide, which blocks the peptidyl transferase reaction on ribosomes, also sensitises cells to ricin and abrin which may indicate that under normal conditions the toxins compete for binding sites within the Golgi-network with newly synthesised proteins or that an enzymatic processing step is required for A-chain translocation (Sandvig *et al.*, 1986).

Strong evidence for translocation from the trans-Golgi network comes from the finding that a hybridoma cell secreting anti-ricin antibodies is resistant to ricin (Youle and Colombatti, 1986). It is postulated that the antibodies inactivate the ricin during transport through the Golgi-network. Only about 1% of the internalised ricin is

translocated to the cytosol which is in accordance with only 5% reaching the Golgi-network (van Deurs *et al.*, 1987b).

1.8 ANTI-VIRAL PROPERTIES OF RIBOSOME-INACTIVATING PROTEINS

Ribosome-inactivating proteins have long been known to have anti-viral properties but the exact mechanism remains unclear. Tomlinson *et al.* (1973) demonstrated that extracts from the leaves of *P. americana* inhibited infection of *Chenopodium quinoa* by cucumber mosaic virus and of monkey kidney cells and embryonated eggs by influenza virus. Leaves of *P. americana* are now known to contain the type I ribosome-inactivating protein called pokeweed antiviral protein (PAP) (Obrig *et al.*, 1973 and Irvin *et al.*, 1980). Animal viruses and bacteriophages alter the membranes of the host both structurally and functionally, leading to a membrane leakiness (Carrasco, 1978). This may therefore allow entry of the type I ribosome-inactivating protein to the cytoplasm whereas the uninfected cell is usually impermeable. Owens *et al.* (1973) postulated that the *P. americana* extract was acting on the host cell machinary and not on the virus *per se*.

Foa-Tomasi *et al.* (1982) studied the anti-viral activity of PAP on polio virus and herpes simplex virus I infection of Hep-2 cells. PAP reduced viral yield, inhibited plaque formation and inhibited protein synthesis more in infected cells than uninfected cells. Fernandez-Puentes and Carrasco (1980) have demonstrated that viral infection permeabilises mammalian cells to protein toxins and therefore it seems probable that anti-viral activity is a consequence of the action of PAP on the host cell machinery in the permeable infected cell.

Abrin and ricin reduced local lesions by tobacco mosaic virus when applied to the tobacco leaf in an inoculum containing the virus and carborundum (Stevens *et al.* 1981). Stevens and co-workers reported that reduction of the interchain disulphide bond in abrin abolished anti-viral

activity. Loss of activity is likely to be due to instability of the reduced protein.

Currently there is an extensive search for an inhibitor of human immunodeficiency virus (HIV). Recently trichosanthin, a type I ribosome-inactivating protein was found to selectively inhibit HIV replication in acutely and chronically infected macrophages and T-cells (McGrath *et al.*, 1989). Healthy cells survived treatment but in cells infected with HIV the viral RNA was apparently specifically degraded whereas the host RNAs were unaffected. This suggests that the anti-viral activity may be a distinct property from *N*-glycosidase action on host ribosomes. Hybrids of ricin A-chain and of PE have been shown *in vitro* to be effective immunotoxins against HIV infected cells (Till *et al.*, 1988 and Chaudhary *et al.*, 1988).

1.9 ANTI-TUMOUR PROPERTIES OF THE TOXIC LECTINS

Anti-tumour activity of abrin and ricin was noted as early as 1951. Since then several laboratories have studied the effects of the toxic lectins on animal tumours and neoplastic cell lines (Olsnes and Pihl, 1982 and Shionoya *et al.*, 1982). The toxic lectins exert their effect through inhibition of protein synthesis which is different to the mechanism of action of other chemotherapeutic agents such as vinblastin, methotrexate and cyclophosphamide. In several instances abrin and ricin were found to be more effective as cancerostatic agents than the drug most commonly used to treat the tumour. In addition, in combined therapy where ricin or abrin were administered in conjunction with a chemotherapeutic agent, a synergenaic effect was seen (Walker *et al.* 1984). Phase I studies of ricin as a cancerostatic agent have been carried out (Fodstad *et al.*, 1984).

The mechanism by which these toxic lectins can suppress tumour growth may be in part due to the direct cytotoxic effects on tumour cells and stimulation of host immunity with non-specific suppression of tumour cells. The binding of a few

molecules of abrin or ricin to a tumour cell may be insufficient for inactivation but may cause increased destruction of toxin-coated cells by the reticuloendothelial system. APA enhances the natural cytotoxicity of peripheral blood lymphocytes *in vitro* and in humans with nasopharangeal carcinoma (Won *et al.*, 1988). Certain tumour cells have been shown to require fewer molecules of toxin to be bound for cytotoxicity (Chan *et al.*, 1985).

Abrin has been used as the positive control in human tumour clonogenic assays for comparison of cytotoxic effects of therapeutic agents (Salmon *et al.*, 1983)

1.10 IMMUNOTOXINS

1.10.1 Overview

An immunotoxin is a cytotoxic agent which consists of a cell-binding moeity, linked via a chemical cross-link, a peptide linker or disulphide bond, to a toxic moeity. The cell-binding moeity may be an antibody, or antibody fragment, or a hormone which has selectivity for a particular cell type. The toxic moeity may be a holotoxin, an A-chain or a single-chain ribosome-inactivating protein. Immunotoxins therefore combine ligand specificity with the exquisite toxicity of the toxin.

Despite the conceptual simplicity of immunotoxins they are complex molecules and each component continues to be studied and improved. Immunotoxins have been extensively reviewed (see Frankel, 1990, Lord *et al.*, 1989, Blakey *et al.*, 1988, Pastan *et al.*, 1986 and Vitetta and Thorpe, 1989) and only the salient points will be mentioned here.

1.10.2 The ligand portion of immunotoxins

Antibodies

Antibodies or fragments thereof have been most frequently utilised as the ligand portion of immunotoxins. The specificity

of monoclonal antibodies can be extremely high although non-neoplastic cells of the same lineage may also be a target. Whole antibodies are large immunogenic molecules and therefore may penetrate solid tumours poorly. Fab' and $F(ab')_2$ fragments are less immunogenic, lack Fc receptors and therefore do not bind to reticuloendothelial cells, and are smaller. However, the antibody fragments are more rapidly cleared from the body and the monovalent Fab' fragments bind with reduced avidity compared to the bivalent or polyvalent counterparts.

Non-antibody carriers

Non-antibody carriers, such as the interleukins and epidermal growth factor, have been used as the ligand in immunotoxins. Generally these ligands will also bind to normal cells but since many neoplastic cells express large amounts of the receptor compared to the normal cell and the normal cells that are killed can be replaced from progenitor cells which lack the receptor, applications continue to be sought.

1.10.3 The toxin portion of antibodies

The extreme toxicity of the toxic lectins has made them the focus of interest in immunotoxin research. The toxin is modified by removal of the B-chain, or the blocking of the galactose binding sites, and specificity is conferred by the addition of a specific ligand. An attractive feature of the toxic-lectins is that resting cells are susceptible to the action of ribosome-inactivating proteins. The single-chain ribosome-inactivating proteins naturally have low toxicity but can be rendered specifically cytotoxic by linkage to a ligand moeity. Bacterial toxins such as diphtheria toxin and *Pseudomonas exotoxin* A have also been extensively utilised to generate immunotoxins.

1.10.4 Preparation of immunotoxins

A variety of linkers have been used to join ligands, with a free thiol group, to the A-chains of the toxic lectins, the holotoxins or single-chain ribosome-inactivating proteins. In the case of the holotoxins the galactose binding sites are stearically hindered by the ligand and are selected by failure to bind to galactose, or have a chemically reactive group covalently attached in the vicinity of the binding sites. For review see Cumber *et al.* (1985).

1.10.5 Cytotoxic properties of immunotoxins *in vitro*

Immunotoxins containing the A-chain of ricin or abrin or the single-chain ribosome-inactivating proteins can be highly specific because the toxin portion does not contribute to cell binding. However, these immunotoxins show a wide degree of cytotoxic potency. It is now generally accepted that the binding affinity of the ligand plays a major role in determining efficacy. A ligand with high affinity generally gives high potency to the immunotoxin. The receptor for the ligand is also important. A receptor that is rapidly internalised is preferential. The routing of the conjugate after internalisation may be disadvantageous e.g. to lysosomal compartments. Immunotoxins constructed with the A-chain of diphtheria toxin are consistently less effective than those constructed with the A-chain of ricin or abrin. This has led to the idea that the toxic lectin A-chains contain a translocation domain, lacking in diphtheria toxin A-chain, that is necessary for transport to the cytosol.

Virtually without exception, the potency of an immunotoxin containing a holotoxin is exceptional but the non-specific binding associated with the B-chain makes their use *in vivo* problematical. The blocked forms of these immunotoxins have been shown to have potent and specific cytotoxicity *in vitro*.

Of importance is that these immunotoxins have faster kinetics of cell killing which will be of particular relevence *in vivo*.

1.10.6 Cytotoxic properties of immunotoxins *in vivo*

Many of the ribosome-inactivating proteins are glycosylated and the B-chains of abrin and ricin are highly glycosylated (Kimura *et al.*, 1988). The mannose- and fucose-containing oligosaccharides are recognised by cells of the reticuloendothelial system. Intact ricin has been used to selectively deplete Kuppfer cells in rodents (Zenilman *et al.*, 1988). Therefore immunotoxins containing native glycosylated ribosome-inactivating proteins are rapidly cleared from the blood and tissues, and liver damage can occur. Deglycosylation of the ribosome-inactivating protein, or the A-chain, has been shown not to alter enzymatic ability (Blakey *et al.*, 1987 and Foxwell *et al.*, 1987). Blocking of the galactose binding sites, commonly performed using asialofetuin affinity labels which are rich in galactose, would therefore only increase liver toxicity. In immunotoxins where the B-chain was deglycosylated, the potentiating effect of the B-chain was reduced by 5 to 50 fold.

From studies in tumour models and in rodents the features desirable in an immunotoxin have become apparent. Ideally the immunotoxin should be highly specific and highly cytotoxic with fast kinetics of killing. The immunogenicity should be low, the half life in sera and tissues should be high and the linkage between the toxic and lectin moeity should be stable. The immunotoxin should be unglycosylated and 100% homogeneous.

1.11 AIMS OF THE PROJECT

The toxic lectins are a fascinating group of proteins. Their extreme potency has made this group of plant proteins of interest to scientists from diverse fields. Ricin in particular has been used as a model, or control, in studies of cell

binding, endocytosis, intracellular transport, protein synthesis and kinetic studies of cell death. Latterly, the use of ribosome-inactivating proteins in the development of anti-cancer agents has demanded more extensive study of this group of proteins.

The initial aim of this project is to clone an abrin-related ribosome-inactivating protein. The only type II ribosome-inactivating proteins sequenced to date are the ricin toxins and the related RCA. It is of interest to compare the primary sequence of other type II ribosome-inactivating proteins and agglutinins since only slight differences significantly alter cytotoxicity and quaternary structure. The abrin toxins are of particular interest as the A-chains are acidic at physiological pH, unlike ricin A-chain and the type I ribosome-inactivating proteins which have typically basic pI values.

It is apparent that the microheterogeneity found between the ribosome-inactivating proteins can lead to problems in interpreting results using protein isolated from plant tissue. The ideal source of a ribosome-inactivating protein is therefore of recombinant nature. The production of large quantities of homogeneous protein is desirable for the study of ribosome-inactivating proteins and in the development of therapeutic agents.

The cloning and expression of an abrin A-chain has application to the field of immunotoxins. Abrin A-chain immunotoxins are more potent than ricin A-chain immunotoxins by 2-7 fold (Sivam *et al.*, 1987 and Blakey *et al.*, 1987b) and human melanoma cell lines have been shown to be more sensitive to abrin immunotoxins than the equivalent ricin immunotoxin (Forrester *et al.*, 1984 and Blakey *et al.*, 1987a). There is little cross reactivity between ricin and abrin therefore immunotoxins containing abrin can be used as an alternative or in conjunction with ricin immunotoxins. The role of the B-chain in determining the efficiency of immunotoxins has become evident and the cloning of a type II ribosome-inactivating

protein is therefore of particular interest to immunotoxin research.

The recent finding that trichosanthin could block the replication of HIV (McGrath *et al.*, 1989) stimulated interest in other ribosome-inactivating proteins as anti-HIV agents in treatment of AIDS. Abrin A-chain was an attractive candidate as it is highly toxic *in vitro*, does not cross react with antibodies against trichosanthin, and the acidic pI of the abrin A-chain may be of significance *in vivo*. Genelabs Incorporated (Redwood City, California, USA) had analysed abrin A-chain (Inland Laboratories) but the preparation contained levels of B-chain sufficient to cause cytotoxicity to uninfected T-cells. The A-chain could not be purified sufficiently for use *in vivo*. The initial aim of this project, to clone an abrin-related gene, was therefore in accordance with a project at Genelabs Inc. to obtain a recombinant abrin A-chain for trial as an anti-HIV agent. The cloning and sequencing of an abrin-related gene was carried out in the laboratories of Genelabs Inc. in colaboration with Dr. M. Piatak. The expression and purification of recombinant A-chain and the expression of B-chain *in vitro* was carried out at Warwick University.

2. MATERIALS AND METHODS

2.1 SUPPLIERS AND REAGENTS

Aldrich Chemical Co. Ltd.: isoamylalcohol; Tween-20

Amersham International plc, Amersham, Buckinghamshire, UK: *E. coli* polymerase I (Klenow fragment); Hybond C nitrocellulose membranes; Hybond N nitrocellulose filters; human placental ribonuclease inhibitor; lambda DNA; radiochemicals; restriction enzymes; T4 polynucleotide kinase

Anderman and Co. Ltd, London Road, Kingston-upon-Thames, Surrey, UK: Schleicher and Schuell NA45 membrane

BDH Chemicals Ltd, Poole, Dorset, UK: acrylamide; chloroform; ammonium acetate; ammonium persulphate; caesium chloride; calcium chloride; dimethyl formamide; ethanol; formaldehyde; glucose; isopropanol; magnesium sulphate; polyethylene glycol; potassium acetate; sodium dodecyl sulphate; sucrose; trichloroacetic acid; Tris base ; Triton X-100; urea

Beckman RIIC Ltd, High Wycombe, UK: non-aqueous scintillant, Quickseal tubes

Biorad Laboratories Ltd, Watford, Hertfordshire, UK: Biogel P-6; Biogel P-2; low-range SDS-PAGE molecular weight standards; prestained SDS-PAGE standards; SP6 RNA polymerase

Boeringer Mannheim, Lewes, East Sussex, UK: calf intestinal alkaline phosphatase; deoxynucleotides; dithiothreitol; random primed DNA labelling kit; ribonucleotides

Difco Laboratories, Detroit, Michigan, USA: agar; Bacto-tryptone

Eastman Kodak, Rochester, New York, USA: Kodabrome IIRC paper; Panatomic-X photographic film

Fisons Scientific Apparatus, Loughborough, Leicestershire, UK: ammonium chloride; ammonium solution; ammonium sulphate; ß-mercaptoethanol; di-sodium hydrogen phosphate; glacial acetic acid; magnesium chloride; phenol; potassium chloride; sodium acetate; sodium chloride; sodium dihydrogen phosphate; sodium hydroxide

Fuji Photo Film Co. Ltd, Japan: X-ray film (RX)

Gibco-BRL Ltd, Uxbridge, Middlesex, UK: kilobase DNA ladder; restriction enzymes; SP6 RNA polymerase; T4 DNA ligase; T7 DNA polymerase

Ilford Ltd, Mobberly, Cheshire, UK: FF contrast developer

Inland Laboratories, Austin, Texas, USA: native abrin A; native abrin C; native *Abrus precatorius* agglutinin, ricin B-chain

Institute of Cancer Research, Sutton, Surrey, UK: affinity purified rabbit anti-abrin C A-chain IgG

Imperial Chemical Industries, Alderly Park, UK: recombinant ricin A-chain

Johnson Matthey, Materials Technology, Royston, Hampshire, UK: silver nitrate

Josman Laboratories, Napa, California, USA: rabbit anti-abrin serum

Nucleotide Collective Services, Genelabs Incorporated, Redwood City, California, USA: oligonucleotides

Paterson Products Ltd, Borehamwood, Hertfordshire, UK: Acutol FX-14 developer

Perkin Elmer Cetus, Norwalk, Conneticut, USA: GeneAmp™ DNA amplification reagent kit; AmpliTaq™

Pharmacia Ltd, London, UK: 5' cap ($^{7me}_{5}G_{ppp}G_{5}OH$); M13 universal primer; plasmid pKK233-2; protein A Sepharose; ultrapure dioxynucleotides;

Pierce Chemical Company, Rockford, Illinois, USA: aminoethylated spherical polyacrylamide beads-immobilised lactose

Poloroid Corporation, Cambridge, Massachusetts, USA: polaroid 667 and 665 film

Premier Brands Ltd, Birmingham, UK: dried fat milk powder

Prolabo, Paris, France: boric acid; ethylenediaminetetraacetate; glycerol

Promega-Biotech, Madison, Wisconsin, USA: anti-rabbit IgG (H-L) alkaline phosphatase conjugate; 5-bromo-4-chloro-3-indolyl phosphate; nitroblue tetrazolium; rabbit reticulocyte lysate (nuclease treated)

Sigma Chemical Co. Ltd, Poole, Dorset, UK: agarose; amino acids; ampicillin; asialofetuin; ß-D-thiogalactopyranoside; Blue Sepharose CL-6B; bovine serum albumin; bromophenol blue; 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; cetyltrimethylammonium chloride; dimethylsuphoxide; ethidium bromide; ficoll 4000; gelatin; hydroxyquinilone; isopropanol; lysozyme; maltose; mineral oil; 4-mopholinepropane sulphonic acid; N,N,N,N'-tetramethylenediamine; NZ Amine A;

phenylmethylsulphonyl fluoride; polyvinylpyrollidone; ribonuclease A; rubidium chloride; spermidine; tetracycline

Stratagene, La Jolla, California, USA: *E. coli* host strains (JM109, XLI-Blue, NM554, PE2392, LE392); Gigapack II Gold packaging extract; Lambda DASHII/*EcoRI* cloning kit; predigested Lambda ZAPII/*EcoRI* cloning kit; vector pBluescript SK+

Unipath Ltd, Basingstoke, Hampshire, UK: yeast extract

United States Biochemical Corp., Cleveland, Ohio, USA: Sequenase Kit; T7-GEN *in vitro* mutagenesis kit

Whatman Labscales Ltd, Maidstone, Kent, UK: 3MM No. 1 filter paper; microfibre glass filters

2.2 PREPARATION OF GENOMIC DNA

2.2.1 Purification and concentration of DNA from aqueous solution

Buffering phenol

Solid crystals of phenol were melted in a 65°C water bath and 8-hydroxyquinoline was added to 1% (mass/v). An equal volume of 0.5M Tris-HCl, pH 8.0, was added and the mixture was stirred for 10 minutes at room temperature. The two phases were allowed to separate and the top aqueous phase was removed. Equilibrations with 0.5M Tris-HCl, pH 8.0, were repeated until the phenol phase reached pH 8.0. One quarter volume of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) was added and the phenol was stored at 4°C in the dark.

Phenol extraction

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution. The sample was

vortexed for 10 s. The phases were separated by spinning for 5 minutes at 13,000g and the upper aqueous layer was transferred to a new tube.

Ethanol precipitation

One tenth volume of 3M sodium acetate, pH 5.2, and 2 volumes of ethanol were added to the DNA solution. The sample was vortexed briefly then placed on dry ice for 10 minutes or at 20°C overnight. The DNA was pelleted for 10 minutes at 4°C. The ethanol supernatant was removed and 1ml of 70% ethanol at room temperature was added. The DNA was pelleted and the supernatant was aspirated. The pellet was dried in a desiccator then dissolved in an appropriate volume of water or in TE buffer for storage.

If the DNA solution contained high salt concentrations it was diluted to reduce NaCl or sodium acetate concentrations to less than 0.5M before addition of ethanol.

Isopropanol precipitation

In general, a one half volume of isopropanol was substituted for ethanol in precipitations if the volume of DNA solution was larger than 0.7ml.

2.2.2 Purification of genomic DNA from plant tissue

Overview

Polysaccharide contamination, which inhibits many enzymes commonly used in cloning procedures, is the most common problem affecting plant DNA purity. Cetyltrimethylammonium bromide (CTAB) complexes with polysaccharides and proteins at high salt concentrations (>0.5M) and both groups of contaminating molecules are efficiently removed in chloroform extraction. At less than 0.5M NaCl the nucleic acid complex will precipitate. Wide bore pipettes were used, with minimal stirring of the plant DNA solutions, to avoid shearing of high molecular mass DNA.

Preparation of genomic DNA from A. precatorius leaf tissue

Approximately 10g of frozen leaf tissue was ground to a fine powder in a prechilled mortar and pestle. B-mercaptoethanol was added to 2% (v/mass) followed by an equal volume of 2 times extraction buffer (2% (mass/v) CTAB, 100mM Tris-HCl, pH 8.0, 20mM EDTA, 1.4M NaCl). The slurry was heated to 50^{o}C in a 55^{o}C water bath with very gentle stirring. An equal volume of chloroform: isoamylalcohol (24:1) was added and mixed by gentle rotation. The phases were separated by centrifugation at 13,000g for 10 minutes at room temperature. The aqueous phase was recovered and chloroform extraction was repeated. A one tenth volume of 10% (mass/v) CTAB, 0.7M NaCl was added and the extraction was repeated a third time. To convert the CTAB-DNA complexes to DNA-Na complexes 2 volumes of 1% (mass/v) CTAB, 50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% (v/v) B-mercaptoethanol were added and the DNA was allowed to precipitate for 30 minutes at room temperature. The DNA was spooled onto a glass rod, allowed to drain, then transferred to 40ml ice-cold 70% ethanol, 0.1M sodium acetate. The DNA was pellated for 3 minutes at 13,000g and the ethanol precipitation was repeated. The DNA pellet was washed in 95% ethanol, air dried and resuspended in 10ml TE buffer at 55^{o}C in a water bath.

2.3 RESOLUTION AND RECOVERY OF DNA FRAGMENTS

2.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separating, identifying and purifying 0.1 to 25kb DNA fragments. Typically agarose concentrations of 0.8% were used to separate larger DNA fragments (>10kb), 1% to separate 0.5-10kb fragments and 1.2% to resolve small DNA fragments (0.1-0.5kb).

Electrophoresis was carried out at 10 volts/cm of gel except for the resolution of large DNA molecules (>10kb) when the applied voltage was low (1 volt/cm of gel).

The desired amount of agarose was added to a volume of 1 X TAE buffer (40mM Tris-acetate, pH 8.5, 2mM EDTA) and melted in a microwave oven. The gel was cast, placed in a horizontal electrophoresis tank and covered with 1 X TAE buffer.

DNA samples were prepared by the addition of the appropriate amount of 10X loading buffer (10X TAE, 50% (v/v) glycerol, 0.01% (mass/v) bromophenol blue) and loaded. Voltage was applied as required and electrophoresis was monitored by the migration of the dye front.

DNA was stained by placing the gel in 0.5μg/ml ethidium bromide in water with gentle agitation for 10-30 minutes and visualised under a long wave UV light source.

2.3.2 Isolation and purification of DNA fragments from agarose gels

DNA was resolved by gel electrophoresis. A piece of NA45 membrane was inserted into the gel ahead of the fragment of interest. Electrophoresis was resumed until the DNA had run onto the membrane.

The DNA was eluted in 50mM arginine, pH 9.0, 1M NaCl for 20 minutes at 65^{o}C with occasional shaking. The membrane was removed and the DNA solution was extracted 3 times with phenol, followed by ethanol precipitation. The DNA pellet was taken up in an appropriate volume of water.

2.4 RESTRICTION ENDONUCLEASES

2.4.1 Digestion of DNA with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubation of the enzyme(s) with the DNA in appropriate

reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction varied depending upon specific application.

All restriction enzymes were obtained from commercial sources, and the appropriate supplied 10X reaction buffer was employed in all cases. All incubations were at 37°C, except for *SmaI* digestion which was carried out at 30°C.

Typically 0.1-4µg of DNA in water or TE buffer was incubated with 2µl of the appropriate 10X restriction endonuclease buffer and 1ul of enzyme in a 20µl reaction volume for a minimum of 1 hour at 37°C. For further enzymatic manipulation of the DNA, the reaction was stopped by heating to 75°C for 15 minutes and the DNA was purified by extraction with phenol and ethanol precipitation.

2.5 PROTOCOLS FOR MODIFYING AND RADIOLABELLING NUCLEIC ACIDS

2.5.1 Radioisotopes used to manipulate nucleic acids

[^{32}P] was used to radioactively label nucleic acids. [^{32}P]-labelled NTP and dNTP precursors were purchased commercially, at specific activities of >110TBq/mmol for [α-^{32}P]-dCTP, [γ-^{32}P]-dATP and [α-^{32}P]-UTP.

Deoxyadenosine 5'-[α-^{35}S] triphosphate, at a specific activity of >37 TBq/mmol, was used for all the dideoxy sequencing procedures.

Measuring radioactivity

A known volume (typically 1µl) of a reaction mixture containing radioactive precursors was spotted onto a glass microfibre filter in duplicate, and allowed to air dry. The second filter was placed in 50ml of ice cold 10% trichloroacetic acid (TCA) solution and stirred gently for 30 minutes. The filter was then transferred to 50ml of 5% TCA at 100°C and boiled for 5 minutes. The filter was washed 3 times

with ice cold 5% TCA and dried under a heating lamp. The filters were placed in separate vials containing 4ml of a non-aqueous scintillation fluid and the amount of radioactivity present was measured in a liquid scintillation counter. The incorporation of radioactivity into the nucleic acid was determined from the ratio of total radioactivity (unwashed filter) to incorporated radioactivity (acid washed filter).

Removal of unincorporated dNTP precursors

For DNA greater than 100bp column chromatography on Biogel P6 was employed. The resin was swelled by adding excess water to Biogel P6, and boiling in a microwave oven for 1 minute. After cooling to room temperature, excess water was removed and replaced with fresh. This was repeated 4 times. The swollen resin was stored at 4°C.

A 1ml disposable syringe was plugged with siliconised glass wool and filled with the pre-swollen resin. The syringe was placed in a polypropylene tube suitable for a bench top centrifuge and spun for 3 minutes at 3000 rpm to pack the column. The radioactive sample was diluted to 100µl with water and loaded onto the column. The column was respun in a clean polypropylene tube. A 100µl volume of water was applied, the column was spun as before, and the sample was recovered from the polypropylene tube.

For oligonucleotides Biogel P2 was used in 0.5X TE buffer and the column was washed in 0.5X TE instead of water.

P6 spin columns were also used to remove excess salt from DNA samples. The sample was recovered in water and then taken down to dryness under vacuum. The DNA was resuspended in a small volume of the buffer of choice.

2.5.2 DNA dependent DNA polymerases

Labelling by oligonucleotide-primed synthesis

Uniform labelling of DNA by "random priming" was the method employed for the radiolabelling of DNA to be used as

sequence-specific probes for screening libraries and genomic DNA Southern blots.

The method of "random primed" DNA labelling is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is synthesized from the 3' OH termini of the hybridized hexanucleotide primer using Klenow enzyme. Labelled dCTP present in the reaction mixture is incorporated leading to a uniform degree of labelling along the length of the input DNA. The input DNA serves solely as the template and is not degraded during the reaction.

A commercially available "Random Primed" DNA Labelling Kit was used (Boehringer Mannheim) and the manufacturer's protocol was followed.

Briefly, 0.1-2μg DNA was heated to 95°C for 10 minutes and subsequently cooled on ice. To the denatured DNA, 3μl of a 0.5mM each dATP, dGTP, dTTP mixture, 2μl of a hexanucleotide mix in reaction buffer, 5μl [α-^{32}P]-dCTP and 1μl of Klenow were added in a final volume of 20μl. The mixture was incubated for 30 minutes at 37°C and the reaction stopped by heating to 65°C for 10 minutes.

Labelling 5' ends of DNA

Refer to T4 polynucleotide kinase (section 2.5.4).

Repairing 3' or 5' overhanging ends

The Klenow fragment of *E. coli* DNA polymerase I was employed to repair 3' or 5' overhanging ends.

To end-fill 5' over-hanging ends 0.1-4μg of DNA was digested in a 20μl reaction volume. One μl of 0.5mM dNTP (0.5mM of each dNTP) was added followed by 1μl of Klenow. The reaction mix was incubated at 30°C for 15 minutes, then heat inactivated at 75°C for 10 minutes.

To blunt back 3' overhanging ends or to repair DNA with both 3' and 5' overhanging ends, the reaction mix was incubated

at 37°C for 5 minutes, followed by incubation at room temperature for 20 min and then 20 minutes on ice.

T7 DNA Polymerase

T7 DNA polymerase is a highly processive enzyme incorporating thousands of nucleotides before dissociation from the template, and does not perform strand displacement synthesis. Cloned T7 DNA polymerase was used for *in vitro* mutagenesis (section 2.12.3).

2.5.3 DNA-Dependent RNA polymerases

SP6 RNA polymerase

SP6 RNA polymerase is highly specific for its own promoter consisting of a 20bp sequence. Transcription is rapid and highly processive.

SP6 RNA polymerase was employed for *in vitro* transcription of cloned DNA inserted into the pSP64-T vector.

2.5.4 Phosphatases and kinases

Calf intestinal alkaline phosphatase

Calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylate the 5' termini of DNA prior to labelling with [γ-^{32}P]-ATP and T4 polynucleotide kinase, and for dephoshorylation of 5' termini of vector DNA to prevent self ligation.

For a 50μl reaction volume, 1-20pmol DNA termini were incubated in 20mM Tris-HCl, pH 8.0, 1mM $MgCl_2$, 1mM $ZnCl_2$ and 0.1u CIAP for 30 minutes at 37°C. The reaction was stopped by heating to 75°C for 10 minutes.

T4 polynucleotide kinase

T4 polynucleotide kinase (PNK) catalyses the transfer of the terminal (γ) phosphate of ATP to the 5' hydroxyl termini of

DNA and was employed for labelling 5' ends or for phosphorylating oligonucleotide primers.

For a 30µl reaction volume, 1-50pmol dephoshorylated DNA 5' ends was incubated in 50mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 5mM DTT, 150µCi [γ-^{32}P]-ATP, 50µg/ml BSA and 20 units of T4 PNK at 37°C for 60 minutes. The reaction was stopped by heating to 75°C for 10 minutes.

For phosphorylating oligonucleotides, 1-10µg DNA was incubated in the above reaction mix with 1mM ATP replacing [γ-^{32}P]-ATP.

2.5.5 DNA ligases

T4 DNA ligase catalyses the repair of single stranded nicks in duplex DNA and joins duplex DNA fragments with cohesive or blunt ends.

For a 15µl reaction volume, 25-100ng of each component DNA was incubated in 40mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 10mM DTT, 0.5mM ATP, 50µg/ml BSA and 10 units of T4 DNA ligase for 2-16 hours at 16°C. The reaction was stopped by heating to 75°C for 10 minutes.

2.6 CONSTRUCTION OF HYBRID DNA MOLECULES

To construct new DNA molecules, the starting DNAs were treated with appropriate restriction endonucleases and other enzymes if necessary. The individual components were purified by agarose gel electrophoresis, combined and treated with DNA ligase. The products of the ligation mixture were introduced into competent *E. coli* cells (section 2.10.3) and transformants were selected by an appropriate genetic selection. DNA was prepared from the colonies or plaques and analysed by restriction endonuclease mapping.

2.7 POLYMERASE CHAIN REACTION

2.7.1 Basic principle of PCR

Introduction

The polymerase chain reaction (PCR) is a cyclic procedure for *in vitro* enzymatic amplification of a specific segment of DNA. The reaction uses two oligonucleotide primers flanking the DNA sequence of interest and hybridising to opposite strands. The primers are orientated such that synthesis by Taq DNA polymerase extends across the segment of target DNA. Each new strand generated can act as a template in subsequent DNA synthesis. Each cycle of amplification consists of strand denaturation, primer annealing and enzymatic extension. The amount of target DNA doubles with each completed cycle.

Basic protocol

A typical PCR mixture consisted of 1µg of template DNA, 100pmoles of each primer (or primer pool), 200µM each dNTP, 2.5 units of Amplitaq™ DNA polymerase, 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$ and 0.01% (mass/v) gelatin in 100µl total volume, with 100µl mineral oil added.

Unless otherwise specified a cycle consisted of denaturation for 1 minute at 94°C, annealing for 2 minutes at 50°C and extension for 2 minutes at 72°C. PCR was carried out for a total of 30 cycles using a Perkin-Elmer thermocycler.

2.7.2 PCR to generate abrin-specific probe

Degenerate primer pools representing all possible coding sequences of an N-terminal and an internal domain of abrin C A-chain (refer to Figure 10, section 3.2.1) were used in a PCR on genomic *A. precatorius* DNA template to generate an abrin specific probe. The optimum temperature for primer annealing was determined empirically.

2.7.3 PCR using linker extended primers

PCR provided an alternative to mutagenesis of target DNA for the introduction of restriction endonuclease sites and additional codons. Linker extended primers were designed which would generate, by PCR on target DNA, the required DNA fragment flanked by suitable unique endonuclease restriction sites and if necessary the sequence of additional codons. The standard design of these linker extended primers were a minimum of 15 bases at the 3' end of the primer which were complementary to the target DNA, a 6-14 base extension encoding the desired restriction endonuclease recognition site and additional codons if required. The linker extended primers were flanked by two G residues at the 5' ends to ensure stabilisation of the DNA duplex. A standard PCR reaction was carried out as described.

2.8 DNA SEQUENCING

2.8.1 Overview

The dideoxy sequencing method, originally developed by Sanger *et al.* (1977), was used to determine DNA sequence information. In practice, the DNA to be sequenced is prepared as a single stranded molecule, and a chemically synthesised oligonucleotide is annealed to the 3' end of the region to be sequenced. The four dNTPs are added (one of which is radiolabelled), and modified T7 DNA polymerase is used to synthesise an oligonucleotide complementary to the template by the formation of phosphodiester bonds between the 3' hydroxyl group of the growing primer and the 5' phosphate of the incoming dNTP. The labelling reaction mix is divided into four aliquots. The addition to each aliquot of one of the four 2',3'-dideoxynucleotides (which lack the 3' hydroxyl group) leads to the termination of the elongating primer chains at each occurence of the included dideoxynucleotide. Each of the

four elongation reactions contains a population of radiolabelled chains, all of which have a fixed 5' end as determined by the primer and a variable 3' end terminating at a specific base. The strands of the molecules are separated by heating and the DNA is electrophoresed on a sequencing gel.

Sequencing Gels

For analysis of DNA sequence close to the primer an 8% polyacrylamide gel was used, and electrophoresis was performed until the bromophenol blue migrated to within 5cm of the bottom of the gel. The standard gel employed contained 6% polyacrylamide, and the bromophenol blue was run to the bottom of the gel. For reading longer oligonucleotide sequence a 4% polyacrylamide gels was used, and the gel was run until the xylene cyanol had run off the gel.

For 100ml of gel mix:

Final [Acrylamide]	4%	6%	8%
40% Acrylamide:Bisacrylamide (30:1)	10ml	6.7ml	5ml
Urea	46.7g	46.7g	46.7g
10XTBE buffer	10ml	10ml	10ml
H_2O to	100ml	100ml	100ml

The gel mix was filtered through a 0.22um filter, then degassed. Polymerisation was initiated by the addition of 250µl of 10% (mass/v) ammonium persulphate and 25µl of TEMED. The gel was cast between two glass plates with 0.4mm spacers.

Electrophoresis was carried out at 40 Watts in 1XTBE (89mM Tris base, 89mM boric acid, 2mM EDTA) buffer. When the dye front had reached the desired position, the gel was fixed for 30 minutes in 10% methanol, 10% acetic acid and dried onto 3MM filter paper under vacuum at 55°C for 30 minutes, then exposed to X-ray film overnight at room temperature.

2.8.2 Sequenase™ sequencing method

DNA sequencing was performed using the commercially available Sequenase™ kit and following the manufacturer's protocol.

Briefly, 0.5 pmoles of primer was incubated with 1μg of DNA in 40mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 50mM NaCl in a 10μl reaction volume for 2 minutes at 65°C and then allowed to cool back slowly to below 30°C. To the annealed template-primer reaction mix, 1μl 0.1M DTT, 2μl labelling mix (1.5μM dGTP, 1.5μM dCTP, 1.5μM TTP), 0.5μl [α-^{35}S]-dATP (10μCi/μl) and 2μl Sequenase™ (diluted 1:8 in TE buffer) were added and the reaction mixture was incubated for 5 minutes at room temperature. When the labelling reaction was complete, 3.5μl was transferred to 2.5μl of each of the termination mixes, prewarmed to 37°C, and incubated at 37°C for 5 minutes. To each termination reaction, 4μl of 95% (v/v) formamide, 20mM EDTA, 0.05% (mass/v) bromophenol blue, 0.05% (mass/v) xylene cyanol was added. The reaction mixes were heated to 80°C for 2 minutes and 3μl was loaded onto a sequencing gel.

For general sequencing, the dGTP termination mixes were employed consisting of 8μM of the selected ddNTP and 80μM of the other three dNTPs. For sequences containing dyad symmetry of dC and dG residues which do not fully denature during electrophoresis, the dITP termination mixes were used consisting of 8μM of the selected ddNTP, 80μM each dATP, dCTP, dTTP and 160μM dITP (except for the ddGTP mix which contains 1.6μM ddGTP). The inclusion of dITP, which forms weaker secondary structure, leads to the denaturation of compressions.

Sequencing plasmid DNA

Plasmid DNA was sequenced directly by including a denaturation step to generate single stranded template. The DNA to be sequenced was taken up in 8μl of water and 2μl of 2M NaOH. The mixture was held at room temperature for 10 minutes, 3μl of 3M sodium acetate, pH 6.0, and 10μl of water were added,

then the DNA was ethanol precipitated. The DNA pellet was taken up in 6µl of water and sequencing was performed as described.

Direct sequencing of PCR products

A standard PCR reaction was performed and the DNA product was purified from an agarose gel. The DNA was lyophilised and taken up with the required amount of primer in a total volume of 8µl of water. The sample was heated to 95°C for 3 minutes, spun briefly to collect condensation, then frozen on dry ice. The DNA/primer mix was thawed between the finger tips, 2µl of annealing buffer was added and the sequencing protocol continued as described above, except that annealing was for 10 minutes at 37°C.

2.9 *ESCHERICHIA COLI*

2.9.1 Media preparation

Minimal media

5 X M9 medium, per litre:

30g Na_2HPO_4
15g KH_2PO_4
5g NH_4Cl
2.5g NaCl

Autoclaved at 15lb/in^2 for 20 minutes

Concentrated media was diluted to 1X with sterile water and the following sterile supplements added per litre:

1ml $MgSO_4.7H_2O$
10ml 20% glucose
0.1ml 0.5% thiamine-HCl, 5% tryptophan, 5% leucine

Rich media

LB medium, per litre:
10g bactotryptone
5g yeast extract
5g NaCl
Autoclaved at 15lb/in^2 for 20 minutes

NZY broth, per litre:
10g NZ Amine A
5g NaCl
2g $MgCl_2.6H_2O$
Autoclaved at 15lb/in^2 for 20 minutes

Solid media

For minimal media plates, 15g of agar in 800ml was autoclaved at 15lb/in^2 for 20 minutes, then sterile concentrated media and carbon source were added. The medium was allowed to cool to 50°C and supplements were added. Approximately 35ml of medium was poured per plate.

For rich media plates, 15g of agar per litre of media was autoclaved at 15 lb/in^2 for 20 minutes. The media was allowed to cool to 50°C, antibiotic was added if required, and 35ml was poured per plate.

Top agar

LB top agar, per litre:
10g bactotryptone
5g yeat extract
5g NaCl
7g agar
Autoclaved at 15lb/in^2 for 20 minutes

Before use, top agar was melted in a boiling water bath and held at 45°C.

2.9.2 Growth in liquid media

Overnight cultures

A single bacterial colony was inoculated into 5-10ml of liquid medium. The culture was grown at 37°C for a minimum of 6 hours to overnight.

Large cultures

Cultures larger than 30ml were inoculated with an overnight culture diluted 1:100 in flasks whose volume was at least 5 times the volume of the culture. Cultures were grown at 37°C, unless otherwise stated, with vigorous shaking (approximately 300rpm).

Monitoring growth

The growth of cultures of *E. coli* were monitored by recording the optical density (OD) at 600nm.

2.9.3 Growth on solid media

Isolating single colonies

Plate streaking: an inoculum of bacteria was streaked across an agar plate using a sterile toothpick. A fresh toothpick was passed once through the first streak. This was repeated once more. Plates were incubated at 37°C until colonies became visible.

Spreading a plate: liquid culture was spread over a plate by pipetting 0.05-1ml of culture onto a dry plate and spreading over the surface of the plate using a glass spreader which had been flamed in ethanol and cooled by touching the agar surface.

Strain storage

For frozen stocks, 1ml of a freshly saturated culture was

added to a vial containing 7% (v/v) dimethylsulphoxide, and stocks were stored at -70°C.

2.9.4 Host strains

JM109

JM109 (recA1, endA1, gurA96, thi, hsdR17, supE44, relA1, lambda$^-$, Δ(lac-proAB), [F', traD36, proAB, laqI^{q}ZΔM15]) was maintained on minimal media plates. JM109 was the host strain used for routine transformations, as feeder cells in the plating of M13 phage and for the cytoplasmic expression of recombinant A-chain.

XL1-Blue

XL1-Blue^{TM} (end A1, hsdR17(r_K^-, m_K^+), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, Δ(lac), [F', proAB, lacI^{q}ZΔM15, Tn10(tet^R)] was maintained on LB media plates supplemented with 15ug/ml tetracycline. XL1-Blue^{TM} was used as the host strain to determine the percentage recombinants in the genomic library generated in the Lambda ZAPII vector and for growth of the recombinant Lambda ZAPII phagemid.

JA221

JA221 (lpp$^-$, hsdR, ΔtrpE5 leuB6 lacY, recA1, [F', lacI^q, lac$^+$, pro$^+$]) was maintained on minimal media plates. JA221 was used for periplasmic expression of recombinant A-chain.

NM554

NM554 (recA1, araD139, Δ(ara-leu)7696, Δ(lac)17A, galU, galK, hsdR, strA, mcrA, mcrB) was maintained on LB media plates. NM554 was used for the generation of genomic libraries.

SDM

SDM (hsdR17, mcrAB, recA1, Δ(lac-proAB), [F', traD36, proAB$^+$, laqI^{q}ZΔM15]) was maintained on minimal media plates.

SDM was used for transformation of methylated M13 DNA following *in vitro* mutagenesis of cloned inserts.

PE2392 and LE392

PE2392 (hsdR514(rk$^-$, mk$^+$), supE44, supF58, Δ(lacIZY), galK2, galT22, metB1, trpR55, (P2) and LE 392 (hsdR514(rk$^-$, mk$^+$), supE44, supF58, (lacIZY), galK2, galT22, metB1, trpR55) were maintained on LB media and were used to determine the percentage recombinants in the genomic libraries generated in the Lambda DASHII vector.

2.10 PLASMIDS

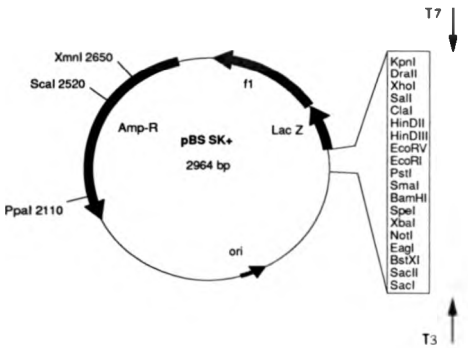
2.10.1 Plasmid vectors

Features of the plasmids employed are described.

BluescriptII SK+

BluescriptII SK+ is a 2964bp phagemid derived from pUC19 (Figure 2). The vector possesses an f1 phage origin, a colE1 origin, T3 and T7 promoters flanking a polylinker containing 19 unique restriction sites, and carries ampicillin resistance. The vector contains a lacZ promoter for blue/white colour selection. The f1 origin of replication allows single stranded DNA rescue via helper phage infection.

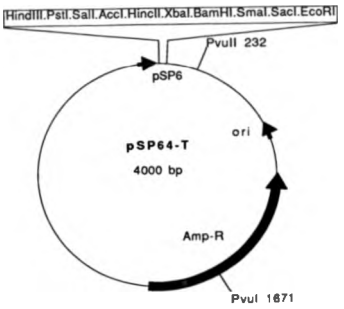
Figure 2 *Bluescript II SK+ plasmid map*



pSP64T

pSP64T is a plasmid derived from pUC13 (Melton *et al.*, 1984) (Figure 3). The vector possesses a SP6 RNA polymerase transcription initiation site, the SP6 RNA polymerase promoter, a polylinker containing 6 unique restriction sites, and carries ampicillin resistance. Between the promoter and polylinker is the sequence encoding the untranslated regions of *Xenopus laevis* B-globulin cDNA (Patient *et al.*, 1983 and Williams *et al.*, 1980) and central to this sequence is a unique *BglII* site. The SP6 RNA polymerase promoter allows *in vitro* transcription from DNA sequences cloned into the *BglII* site, subsequent to vector linearisation using an enzyme with a unique recognition site within the polylinker.

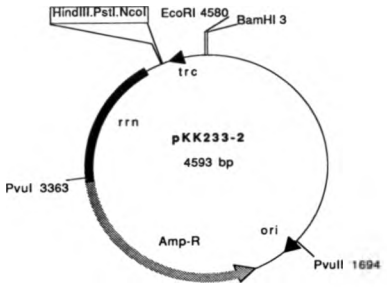
Figure 3 *pSP64-T plasmid map*



pKK233-2

The vector pKK233-2 is a 4593bp plasmid derived from pBR322 and contains the *trc* promoter, the lacZ ribosome binding site, an ATG initiation codon and a rrnB transcription terminator downstream of a multiple cloning site (Figure 4). The plasmid carries ampicillin resistance.

Figure 4 *pKK233-2 plasmid map*



2.10.2 Plasmid DNA preparation

Minipreps of plasmid DNA

A modification of the method described by Holmes and Quigley (1981) was used for the small scale preparation of DNA.

A 10ml overnight culture was pelleted at 3000rpm in a bench top centrifuge. The pellet was resuspended in 180µl of 0.1M Tris-HCl, pH 8.0, 50mM EDTA, 20% sucrose and transferred to an Eppendorf tube. Then 180µl of 4mg/ml lysozyme in 0.1M Tris-HCl, pH 8.0, 50mM EDTA, 20% (mass/v) sucrose was added. After 5 minutes at room temperature, 300µl 10% Triton X-100 was added, the sample was vortexed, then boiled for two minutes and placed on ice for two minutes. Chromosomal DNA was pelleted for 30 minutes in a microfuge and the supernatant containing plasmid DNA was recovered to a fresh tube. A 300µl volume of 7.5M ammonium acetate was added and the sample placed on ice for 20 minutes. The sample was centrifuged for 10 minutes at room temperature. The supernatant was recovered, and the DNA was precipitated with isopropanol. The DNA was resuspended in 50µl TE buffer and 1µl 10mg/ml DNase-free RNase was added.

Caesium chloride/ethidium bromide preparation of plasmid DNA

Cells from a 400ml culture were pelleted in a Sorvall 6X250 rotor at 5000rpm for 5 minutes. The pellet was resuspended in 14.4ml 0.1M Tris-HCl, pH 8.0, 50mM EDTA, 20% sucrose, containing 30mg of lysozyme, and left at room temperature for 5 minutes. A 10ml volume of 10% Triton-X100 was added and the flask was boiled over an open flame for 1 minute followed by 1 minute at 100^{o}C in a water bath. The flask was placed on ice for 2 minutes. The lysed cells were pelleted at 21,000rpm at 4^{o}C in a Sorvall 6X50 rotor for 20 minutes. The supernatant was recovered, 0.5 volumes of 7.5M ammonium acetate was added and the sample was placed on ice for 20 minutes. The solution was centrifuged at 15,000rpm at 4^{o}C in a Sorvall 8X50 rotor for 10 minutes. The supernatant was recovered and DNA

precipitated with isopropanol. The pellet was resuspended in 4ml TE buffer, followed by the addition of 4.5g of caesium chloride and 0.5ml ethidium bromide (5mg/ml). The solution was loaded into a Beckman Quickseal tube which was then heat sealed. The plasmid DNA was banded by centrifugation at 52,000rpm at 20°C in a Vti65 rotor for 16 hours. Covalently closed plasmid DNA was recovered from the gradient using a syringe and needle. The ethidium bromide was removed by extraction four times with isopropanol saturated with TE buffer and caesium chloride. The solution was diluted five fold in TE buffer and ethanol precipitated. The pellet was taken up in 0.5ml TE buffer, the absorbance at 260nm was determined and the DNA was diluted to 1mg/ml with TE buffer.

2.10.3 Introduction of plasmid DNA into cells

Competent cells

Competent cells were prepared by the method of Hanahan (1980). An overnight culture of *E. coli* cells was diluted 1:100 in fresh prewarmed medium and grown at 37°C until the OD_{600} reached 0.48 (approximately 10^7 cells/ml), then placed on ice for 5 minutes. The cells were pelleted at 3000rpm, at 4°C for 5 minutes in an 8X50 Sorvall rotor. The cells were gently resuspended in one third growth volume 100mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 15% (mass/v) glycerol, at pH 5.8 and left on ice for 15 minutes. The cells were repelleted, and resuspended in 1/12.5 growth volume of 10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% gycerol, at pH 6.8. The cells were left on ice for 15 minutes and then divided into 200µl aliquots in prechilled tubes, and frozen at -70°C.

Transformation

To introduce DNA into competent cells, a 200µl aliquot of frozen competent cells was thawed on ice, and one fifth volume of ligation mixture, or 50ng of plasmid DNA, was added and the cells left on ice for 1 hour. The cells were heat shocked at

42°C for 90s, then placed on ice. To allow for the expression of plasmid encoded antibiotic resistance, 800µl of LB medium was added and the tube placed at 37°C for 20 minutes. Aliquots of the transformation culture were plated out on plates containing the appropriate selective medium and incubated for 16 hours at 37°C.

2.11 LAMBDA PHAGE VECTORS

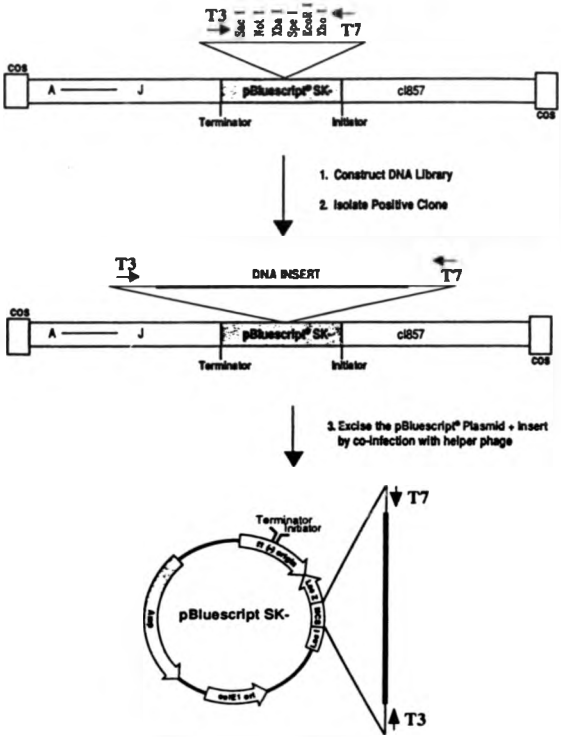
2.11.1 Lambda vectors

Lambda ZAPII

The Lambda ZAPII vector has 6 unique cloning sites that can accomodate DNA inserts from 0-10kbp in length. The vector contains the pBluescript phagemid which allows the insert to be rapidly excised *in vivo* and characterised in a plasmid system (Figure 5).

For *in vivo* excision and recircularisation of a cloned insert, the host strain used must carry the F' episome, which contain the genes for the expression of the bacterial F' pili, and an fI helper phage is required. The proteins from the fI phage recognise the initiator DNA sequence within the Lambda ZAP vector and nick one of the two DNA strands. New DNA synthesis begins and duplicates the DNA downstream of the nicking site until the termination signal within the vector is reached. The single stranded DNA molecule is circularised by the gene II product from the fI phage which recreates a functional fI origin. Signals for packaging of the newly created phagemid are contained within the fI terminator sequence and lead to secretion from the *E. coli* cells. The *E. coli* host cells are killed by heating to 70°C. The "packaged" pBluescript DNA is mixed with fresh host cells and the transfected *E. coli* is plated out on solid media to produce colonies.

Figure 5 *Lambda ZAPII vector map and excision*



Lambda DASHII

The Lambda DASHII vector is a standard lambda vector and can accomodate DNA inserts from 9-23kbp (Figure 6).

Figure 6 *Lambda DASHII vector map*

2.11.2 Plating of phage Lambda

An overnight culture of *E. coli* in LB media, supplemented with 0.2% (mass/v) maltose and 10mM $MgSO_4$, was pelleted at 1000g for 10 minutes then resuspended in 10mM $MgSO_4$ at OD_{600}=0.5. A phage lysate was diluted accordingly in suspension medium (5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml 1M Tris-Cl, pH 7.5, 0.1% (v/v) gelatin) and 0.1ml of diluted phage was added to 0.2ml of host cells. Phage and host cells were incubated at 37^{o}C for 15 minutes to allow for phage absorption. Top agar (3ml) at 45^{o}C was added and mixed gently, then plated out onto a 80mm X 15mm NZY plate. The plates were incubated for 12 hours at 37^{o}C. For 150mm X 15mm plates, 0.6ml of host cells (OD_{600}=0.5 in 10mM $MgSO_4$) and 6.5ml top agar was used per plate.

2.11.3 Growth of phage lambda

Liquid lysate

A single plaque was picked into 0.4ml 20mM Tris-HCl, pH 8.0, 20mM $MgCl_2$ and incubated at 4^oC for 2 hours to allow the phage to elute. To 0.1ml of a saturated culture of host *E. coli*, 0.1ml of eluted phage and 0.1ml 10mM $MgCl_2$, 10mM $CaCl_2$ was added and the mixture incubated at 37^oC for 15 minutes to allow phage absorption. The solution was added to 50ml of NZY medium and shaken at 300rpm for 6 hours at 37^oC or until lysis had occured. Cell debris was removed by centrifugation at 5000g for 10 minutes at 4^oC. The supernatant was decanted and stored in aliquots at 4^oC in the presence of 0.3% (v/v) chloroform.

Plate lysate

Approximately 50,000 lambda phage were plated out onto 150mm X 15mm NZY agar plates and incubated at 37^oC until plaques were clearly visible and approximately 90% of the lawn was lysed. A 10ml volume of suspension medium was pipetted onto the lawn and the plate was left at 4^oC with gentle rocking overnight. The liquid was decanted, the plate was rinsed with an additional 2ml of suspension medium and pooled, and chloroform to 0.5% (v/v) was added and mixed. Cell debris was removed by centrifugation at 5000g for 10 minutes at 4^oC. The supernatant was decanted and stored in aliquots at 4^oC in the presence of 0.3% (v/v) chloroform.

2.12 FILAMENTOUS PHAGE M13

Overview

The filamentous phage M13 was used for site-specific mutagenesis of cloned DNA inserts at internal sites. The commercially available USB T7 GEN™ In Vitro Mutagenesis Kit was used. This method employs an *in vitro* selection procedure in which the mutant strand is selectively methylated by 5-

methyl-dCTP incorporation during second strand synthesis. The non methylated parent strand is subsequently removed by treatment with *Sau3A1*, which nicks only the unmethylated DNA, and exonuclease III which digests the nicked parental strand. The inclusion of *HhaI* in the reaction removes uncopied single stranded DNA. The method is highly efficient (mutation frequencies >75%) and rapid.

2.12.1 Introduction of M13 into cells

Competent cells of an appropriate host strain were prepared. To a 200ul aliquot of competent cells, 100ng of M13 DNA was added and then held on ice for 45 minutes. The cells were heat shocked at 42°C for 90s and then quenched on ice. Cells were plated out onto prewarmed LB plates in 3ml of top agar, containing 40µl of 20mg/ml X-gal in dimethylformamide and 10µl of 20mg/ml IPTG, held at 42°C. Plates were incubated at 37°C for 16 hours.

2.12.2 Preparation of single stranded M13 DNA

A single plaque was picked into 2ml of a 1/100 dilution of an overnight culture of *E. coli* host cells in LB and shaken vigorously at 300rpm for 6 hours at 37°C. The lysate was transferred to an Eppendorf tube and cells were pelleted for 5 minutes at 4°C in a microfuge. The supernatant was taken off with a pipette to a fresh tube and one tenth volume of 20% PEG 6000, 2.5M NaCl was added and the phage were allowed to precipitate for 30 minutes at 4°C. The pelleted cells were saved for the preparation of M13 RF DNA. The phage was pelleted for 10 minutes at 4°C, and the supernatant was removed with a drawn out Pasteur pipette. The phage was resuspended in 150ul of TE buffer and extracted with an equal volume of phenol. The phases were separated by centrifugation for 4 minutes, and the sample was re-extracted with an equal volume of chloroform. Traces of chloroform were removed by 3 sequential ether

extractions. The DNA was recovered by ethanol precipitation, and the pellet was resuspended in 20µl TE buffer.

2.12.3 In vitro mutagenesis

M13 single stranded template containing target DNA was prepared and diluted to 1 pmole/µl. In a 10µl total volume, 5 pmoles of single stranded target DNA was incubated with 2 pmoles of phosphorylated mutagenic primer in 40mM Tris-HCl, pH 7.5, 20mM $MgCl_2$, 50mM NaCl for 5 minutes at 65°C, and then cooled slowly back to room temperature.

To the annealed template/primer mix, 2µl of 100mM Tris-HCl, pH 7.5, 20mM DTT, 5mM dATP, 5mM dGTP, 5mM dTTP, 10mM ATP, 5mM 5-methyl-dCTP was added, the volume was made up to 18µl with water and 2.5 units of T7 DNA polymerase and 5 units of T4 DNA ligase were added. The mix was incubated at 37°C for 60 minutes, then heat inactivated for 10 minutes at 70°C.

To the hemimethylated DNA 5 units of Sau3A and 10 units of *HhaI* were added and the mix was incubated for 45 minutes at 37° C. Fifty units of exonuclease III was then added and the mix incubated for a further 45 minutes at 37°C. The reaction was stopped by heating to 70°C for 10 minutes.

The DNA was transformed into competent SDM *E. coli* cells and then plated out with *E. coli* JM109. Plaques were picked, single stranded DNA was prepared and analysed by DNA sequencing.

2.12.4 Preparation of double stranded M13 DNA

The cells from a 1.5ml phage lysate were pelleted and the supernatant removed. The cells were resuspended in 100µl TE buffer and left at room temperature for 5 minutes. Two volumes of 2M NaOH, 1% (mass/v) SDS were added and the tube placed on ice for 5 minutes. A half volume of 3M potassium acetate, pH 4.8, was added and held on ice for a further 5 minutes. Cell debris was removed by centrifugation at 4°C in a microfuge,

and the supernatant was transferred to a fresh tube. The DNA was recovered by ethanol precipitation and the pellet was resuspended in 20µl TE buffer and 1µl of 10mg/ml DNase-free RNase. The DNA was analysed by restriction endonuclease mapping.

2.13 PREPARATION OF INSERT DNA FROM GENOMIC DNA

2.13.1 Preparation of DNA for ligation

In a 50µl reaction volume, 10µg of genomic *A. precatorius* DNA was digested with *EcoRI*.

Lambda ZAPII/*EcoRI*

The DNA was concentrated by ethanol precipitation and resuspended in TE buffer at 1mg/ml.

Lambda DASHII/*EcoRI*

The DNA was treated with CIAP, concentrated by ethanol precipitation and resuspended in TE buffer at 1mg/ml.

2.14 PRODUCTION OF GENOMIC DNA LIBRARIES

2.14.1 Ligation

Lambda ZAPII

Test ligations were performed to determine the optimum concentration of insert. In a ligation reaction volume of 5µl, 1µg of Lambda ZAPII/*EcoRI* prepared arms were incubated with 50ng, 100ng, 150ng, 200ng or 250ng of insert DNA at room temperature for 1 hour then overnight at 4°C. A control ligation was performed using 0.4µg of the supplied test insert.

Lambda DASHII

For a ligation reaction volume of 5ul, 1ug of Lambda DASHII/*EcoRI* prepared arms was incubated with 100ng, 200ng, 300ng, 400ng or 500ng of insert DNA (CIAP treated) at room temperature for 1 hour followed by overnight at $4^{o}C$. A control ligation was performed using 0.4μg of the supplied test insert.

2.14.2 Packaging

The Gigapack™II Gold Packaging Extract (Stratagene) was employed for generation of recombinant lambda phage.

Sonic extract was thawed on ice. The freeze/thaw extracts were warmed between the fingers until they just began to thaw, 1μl of the ligation mix was added and the tube placed on ice. Immediately, 15μl of sonic extract was added, mixed gently, then briefly spun. The packaging mix was incubated at room temperature for 2 hours. A 500μl volume of SM buffer was added followed by 20μl of chloroform. The contents were spun briefly. The supernatant was titred.

2.14.3 Determining representation

To determine that sequences of interest, i.e. abrin-related DNA sequences, were contained within the packaged library, 1μl of each packaged extract was diluted 1:100 in TE buffer. A standard PCR was carried out using 1μl of the diluted extract as template and 100pmoles of the primer pools employed to generate an abrin-specific probe. The products of PCR were analysed by agarose electrophoresis for the presence of an amplified DNA sequence.

2.14.4 Determining efficiency of ligation and packaging

Lambda ZAPII

To test the quality of the packaged ligation products, 1μl of 500μl packaged reaction was plated with 200μl of XLI-Blue

cells (OD_{600}=0.5) onto NZY agar plates in 3ml top agar, containing 12.5mM IPTG and 3mg/ml X-gal (in DMF). The plates were incubated at 37°C for 6 hours. Background (blue) plaques were less than $1X10^5$ pfu/μg of arms and positive plaques (white) were greater than 50 fold above background. The optimum test ligation was selected and the remaining sample was packaged.

Lambda DASHII

To test the quality of the packaged ligation products, 1μl of each 500ul packaged reaction was serially diluted. The diluted packaged material was plated with 200μl each of OD_{600}=0.5 LE392 and P2392 cells in 3ml of top agar onto NZY agar plates. The percentage recombinants were determined from loss of sensitivity to P2 inhibition. The optimum test ligation was selected based on the highest titre on NM554 cells, and the remaining sample of the selected ligation was packaged.

2.15 SCREENING OF RECOMBINANT DNA LIBRARIES

2.15.1 Plating libraries and transfer to filter membranes

The packaged genomic DNA libraries were plated out onto 150mm X 15mm NZY plates using the host strain NM554 at approximately 50,000 plaques per plate and were incubated overnight. The plates were chilled for 2 hours at 4°C. A nitrocellulose filter was placed onto each plate for 1 minute and were orientated by piercing through to the agar with a needle. A second transfer was performed for 4 minutes with a duplicate filter. The filters were floated on the surface of 0.5M NaOH, 1.5M NaCl for 2 minutes to denature followed by neutralisation for 2 minutes on 1.5M NaCl, 0.5M Tris-HCl, pH 8.0. The filters were then washed in 2X SSC (15mM NaCl, 15mM sodium citrate) for 5 minutes. The filters were blotted onto 3MM paper and air-dried. The filters were baked at 80°C for 2

hours in a vacuum oven prior to hybridisation with radiolabelled probe.

2.15.2 Hybridisation with radioactive probe

The nitrocellulose filters were incubated for 1 hour in 6X SSC, 0.5% (mass/v) SDS, 10mM EDTA, 5X Denhardts solution and 100ug/ml denatured salmon sperm DNA at 65^{o}C. Abrin-related probe was radiolabelled by "random priming" and unincorporated dNTPs were removed. The probe was boiled for 5 minutes then placed on ice. The filters were removed from the prehybridisation solution then incubated in 1ml/filter of 6X SSC, 0.5% (mass/v) SDS, 5X Denhardts solution, 0.01M EDTA, 100µg/ml denatured salmon sperm DNA, 1x10^{5} cpm/ml of radiolabelled probe at 65^{o}C for 14 hours. The filters were removed, excess buffer allowed to drain, and washed in an excess volume of 2x SSC, 0.1% (mass/v) SDS at 65^{o}C for 15 minutes. The wash was repeated. The filters were washed in 1x SSC, 0.1% (mass/v) SDS for 15 minutes at 65^{o}C, followed by a final wash in 0.1X SSC, 0.1% (mass/v) SDS for 15 minutes at 65^{o}C. Excess buffer was allowed to drain, then the filters were placed between plastic wrap, marked with IBI Glo-Juice to orientate and exposed to X-ray film overnight at -80^{o}C.

2.15.3 Purification of hybridisation-positive plaques

Hybridisation-positive regions were cored from the master plate using the wide end of a Pasteur pipette and a small secondary library was plated out. Plaques from the secondary plates were transferred to nitrocellulose filters and screened using the radiolabelled abrin-related probe. An isolated plaque was picked, replated and screened. This process was repeated until the desired hybridisation-positive plaque was pure. A pure plaque was used to prepare a phage lysate. The phage stocks of each positive clone was stored at 4^{o}C.

2.15.4 *In vivo* excision protocol

Recombinant pBluescript plasmid was recovered from positive recombinant Lambda ZAPII clones by *in vivo* excision. Briefly, in a 50ml conical tube, 200µl of OD_{600}=1 XL1-Blue cells were incubated with 200µl of phage stock ($1x10^5$ pfu) and 1µl of R408 helper phage for 15 minutes at 37°C. A 5ml volume of LB medium was added and the mixture was incubated at 37°C for 3 hours with shaking at 300rpm. The sample was heated at 70°C for 20 minutes then pelleted at 4000g for 5 minutes and the supernatant was decanted. To plate the rescued phagemid 10µl of the phagemid supernatant was incubated with 200µl OD_{600}=1 XL1-Blue cells at 37°C for 15 minutes, then aliquots were plated out onto LB plates containing 100µg/ml ampicillin and incubated overnight at 42°C to remove any helper phage and allow colony formation. A single colony was streaked onto a fresh LB plate supplemented with 100µg/ml ampicillin.

Recombinant pBluescript plasmid DNA was prepared and analysed by endonuclease restriction mapping. The inserts were fully characterised by DNA sequencing. Oligonucleotide primers were synthesised as required to extend the DNA sequence analysis.

2.16 EXPRESSION OF RECOMBINANT PROTEINS IN *E. COLI*

The steps involved in the construction of the expression vectors and the recombinant vector maps are presented in the results section (section 3.8).

2.16.1 Cytoplasmic expression

E. coli host strain JM109 was transformed with the recombinant expression vector and a frozen stock was prepared from a positive transformant. A 1 litre volume of minimal media supplemented with 100µg/ml ampicillin was inoculated with 1ml

of an overnight culture of JM109/expression plasmid in minimal media supplemented with 100μg/ml ampicillin. The culture was grown to OD_{600}=0.8 at 37°C with shaking at 300rpm. The culture was shifted to 30°C and induced by the addition of IPTG to 1mM. The culture was grown for a further 3 hours, the OD_{600} was recorded and the cells were pelleted at 7000rpm in a Sorval 8x500 rotor at 4°C for 10 minutes.

The cell pellets were resuspended at 20 OD_{600} units/ml in 100mM Tris-HCl, pH 8.0, 5mM EDTA at 4°C. The suspension was sonicated at an amplitude of 18 microns for 1 minute intervals with cooling on ice until the sonicate became translucent. Cell debris was removed by centrifugation at 8000g for 10 minutes at 4°C and the supernatant was recovered and stored at 4°C.

2.16.2 Periplasmic expression

A 1 litre culture of minimal media supplemented with 100μg/ml of ampicillin was inoculated with 1ml of an overnight culture of JA221 harbouring recombinant expression vector. The culture was incubated at 37°C with shaking at 300rpm until the OD_{600} reached 0.8. The culture medium was supplemented with 2% casaamino acids, then induced with 1mM IPTG. The culture was grown for another 3 hours, then the cells were pelleted at 7000rpm in a Sorvall 6X500 rotor at 4°C for 10 minutes.

Periplasmic proteins were isolated by osmotic shock. The pelleted cells were resuspended in 1X growth volume of 10mM Tris-HCl, pH 7.1, 30mM NaCl, then repelleted. The wash was repeated and the cells were resuspended in one third growth volume of 33mM Tris-HCl, pH7.1, 0.4mM EDTA, 20% (mass/v) sucrose and left at room temperature for 30 minutes. The cells were repelleted as before, then rapidly and vigorously resuspended in one growth volume of ice cold 0.1mM $MgCl_2$. The solution was left on ice for 30 minutes. The shocked cell fraction was pelleted out and the supernatant containing periplasmic proteins was recovered and stored at 4°C. For small volumes (500μl), the sample was concentrated by lyophilisation,

and resuspended in polyacrylamide gel electrophoresis loading buffer prior to analysis. Protein from larger volumes was concentrated by ammonium sulphate precipitation.

2.17 ELECTROPHORETIC SEPARATION OF PROTEINS

The discontinuous buffer system of Laemmli (1970) was employed for protein analysis. Stock acrylamide containing 30% (mass/v) acrylamide and 0.8% (mass/v) bis-acrylamide was used. Resolving gels with a final concentration of 12% or 15% stock acrylamide were employed.

Resolving gel mix

Final [acrylamide]	12%	15%
Stock acrylamide	16ml	20ml
3M Tris-HCl, pH8.8	5ml	5ml
10% (mass/v) SDS	0.4ml	0.4ml
H_2O to	40ml	40ml

Polymerisation was initiated by the addition of 200µl of 10% (mass/v) ammonium persulphate and 20µl of TEMED.

Water-saturated isobutanol was layered on top of the resolving gel during polymerisation. Once the gel was set, the isobutanol was removed by washing with distilled water, and replaced with the polymerising stacking gel mix.

Stacking gel mix

Stock acrylamide	1.3ml
0.5M Tris-HCl, pH6.8	2.5ml
10% (mass/v) SDS	0.1ml
H_2O to	10ml

Polymerisation was initiated by the addition of 50ul of 10% ammonium persulphate (mass/v) and 5µl of TEMED. A gel comb was inserted and the gel allowed to set. Once set, the comb was removed and the gel assembled into a vertical electrophoresis tank, with 25mM Tris-HCl, pH 8.8, 192mM glycine, 0.1% (mass/v) SDS in the top and bottom resevoirs.

For reducing SDS-PAGE, samples for analysis were combined with the appropriate volume of 10X loading buffer (50mM Tris-HCl, pH 6.8, 1% (mass/v) SDS, 10% (v/v) glycerol, 0.05% (v/v) ß-mercaptoethanol, 1% (mass/v) methionine, 0.05% (mass/v) bromophenol blue) and boiled for 5 minutes then quenched on ice. For non-reducing SDS-PAGE the ß-mercaptoethanol was omitted from the loading buffer.

Samples were then loaded and electrophoresed at 40mA until the dye front reached the bottom of the gel.

The molecular weight markers routinely used were pre-stained protein standards or low range protein standards (Biorad) with molecular weights and for autoradiography the [^{14}C]-methylated molecular weight standards were used (Amersham).

	Low Range(Da)	Pre-stained(Da)
Rabbit muscle phoshorylase b	97,400	110,000
Bovine serum albumin	66,200	84,000
Hen egg white ovalbumin	45,000	47,000
Bovine carbonic anhydrase	31,000	33,000
Soyabean trypsin inhibitor	21,500	24,000
Hen egg white lysozyme	14,400	16,000

	Daltons
[^{14}C]-methylated myosin	200,000
[^{14}C]-methylated phosphorylase-b	97,400
[^{14}C]-methylated bovine serum albumin	69,000
[^{14}C]-methylated ovalbumin	46,000
[^{14}C]-methylated carbonic anhydrase	30,000
[^{14}C]-methylated lysozyme	14,300

2.10 DETECTION OF PROTEINS

2.10.1 Detection of proteins in gels

Coomassie blue staining

The gel apparatus was dismantled and the gel was stained in an excess volume of 10% glacial acetic acid, 40% methanol, 0.25% (mass/v) Coomassie Brilliant Blue for 1-3 hours at room temperature. The gel was transferred to 40% methanol, 10% acetic acid and gently agitated until protein bands were clearly visible.

Silver staining

The gel apparatus was dismantled. Gels were incubated in 50% (v/v) methanol for a minimum of 3 hours, then transferred to freshly prepared solution A (21ml of 0.36% (mass/v) NaOH and 1.4ml of 14.8M ammonium solution were combined and added dropwise with stirring to 0.8g of silver nitrate in 4ml of water, then the volume was made up to 100ml with water) for 15 minutes. The gels were washed with copious volumes of deionised water then transferred to solution B (2.5ml 1% (mass/v) citric acid and 0.25ml 38% (v/v) formaldehyde in 500ml of water) for 15 minutes or until protein bands were clearly visible. The gels were then fixed in 10% (v/v) glacial acetic acid, 40% (v/v) methanol for 1 hour.

Autoradiography

The gel was fixed in 30% (v/v) methanol, 10% (v/v) glacial acetic acid for 1 hour at room temperature. The gel was transferred to an excess volume of Amplify™ for 15 minutes at room temperature. The gel was dried under vacuum at 65°C onto Whatman 3MM paper then exposed to X-ray film for 4 hours to overnight at room temperature.

2.18.2 Detection of proteins on nitrocellulose

Western blot

Proteins were transferred to nitrocellulose by a modification of the method of Towbin *et al.* (1979). Gels were equilibrated in transfer buffer (25mM Tris-HCl, pH 8.3, 192mM glycine, 20% (v/v) methanol) for 15 minutes with gentle shaking. The gel was layered with an equal sized piece of nitrocellulose, then placed between 2 pieces of 3MM paper and the felt pads of the Biorad transblot apparatus. The apparatus was assembled and filled with transfer buffer. A 50 mA current was applied for 3 hours.

The apparatus was dismantled and the nitrocellulose was incubated in 50ml of TBST (50mM Tris-HCl, pH 7.5, 0.9% (mass/v) NaCl, 0.1% (v/v) Tween-20 containing 5% (mass/v) fat-free dried milk powder for 1 hour to block non-specific antibody binding sites. The nitrocellulose was transferred to 10ml block solution containing a 1:2000 dilution of rabbit anti-abrin C A-chain antibody and incubated at 37^{o}C for 1-3 hours with gentle shaking. The filter was washed three times in 50ml TBST for 15 minutes.

Alkaline phosphatase development system

The ProtoBlot Alkaline Phosphatase System (Promega) was routinely used for Western blot analysis.

The washed filters were incubated in 15ml TBST containing 2μl anti-(rabbit IgG)-alkaline-phosphatase conjugate for 1 hour at room temperature. The filters were washed three times for 15 minutes in 50ml TBST, then blotted damp dry on a piece of 3MM paper. The damp filters were transferred to 10ml colour development substrate solution prepared by the addition of 66μl of 50mg/ml nitroblue tetrazolium (in DMF) and 33μl of 50mg/ml 5-bromo-4-chloro-3-indolyl phosphate (in DMF) to 100mM Tris-HCl, pH9.5, 100mM NaCl, 5mM $MgCl_2$. When the colour had developed to the desired intensity, the reaction was stopped by

replacing the substrate solution with 20mM Tris-HCl, pH 8.0, 5mM EDTA. The filter was then air dried.

[^{125}I]-protein A development system

After incubation with the primary antibody and washing, the filters were incubated in 10ml TBST containing 1µl [^{125}I]-protein A (kindly provided by Dr P. Richardson, University of Warwick) overnight at room temperature, with gentle shaking.

The filter was then washed 5 times for 5 minutes in 50ml TBST, then placed between plastic film. The filter was marked with IBI Glo-Juice, then exposed to X-ray film for 1 week.

2.19 PURIFICATION OF SOLUBLE RECOMBINANT A-CHAIN

2.19.1 Solubility

Solubility of recombinant proteins was determined by centrifugation at 108,000g for 1 hour at 4°C. The supernatant containing soluble proteins was recovered, and the pellet was washed with 100mM Tris-HCl, pH 8.5, 5mM EDTA, then resuspended in an equal volume to the supernatant of 100mM Tris-HCl, pH 8.5.

2.19.2 Ammonium sulphate precipitation

Concentration of recombinant abrin A-chain was performed using $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to the soluble fraction from cell sonicates (or periplasmic fractions) to 30% (mass/v) slowly at 4°C with stirring. The precipitated proteins were recovered by centrifugation at 15,000g for 15 minutes at 4°C, then resuspended in one tenth precipitation volume of 100mM Tris-HCl, pH 8.5, 5mM EDTA. The sample was filtered through a 0.22µm filter.

2.19.3 Ion-exchange HPLC

The recombinant abrin A-chain was purified using an LKB DEAE-5PW UltraPac column (21.5X150mm) with a Beckman model 344 HPLC system. The column was equilibrated with the starting buffer (10mM NaCl, 5mM sodium phosphate buffer, pH 7.5), and a 5ml volume of sample was applied. The column was washed with the starting buffer at 3ml/minute until the OD_{280} reached the baseline level. The bound proteins were eluted with a gradient from 10mM to 500mM NaCl over 2 hours at 3ml/minute and eluate was collected in 3ml fractions. The fractions were analysed by SDS-PAGE and Western blotting. Fractions containing A-chain were pooled and dialysed against 10mM NaCl, 5mM sodium phosphate buffer, pH 7.5.

2.19.4 Blue Sepharose CL-6B chromatography

Recombinant A-chain was purified to homogeneity by chromatography on Blue Sepharose CL-6B. A 30X1cm column was packed with Blue Sepharose CL-6B resin, pre-swollen overnight in an excess of 100mM NaCl, 5mM sodium phosphate buffer, pH7.5, at a flow rate of 0.275ml/minute. The column was equilibrated with starting buffer (100mM NaCl, 5mM sodium phosphate buffer, pH 7.5) and the baseline for OD_{280} was established, at a flow rate of 0.15ml/minute.

The A-chain sample was applied, and the column was washed at 0.15ml/minute with starting buffer until all non-bound materials had eluted, and the OD_{280} baseline was reached. A gradient to 500mM NaCl, 5mM sodium phosphate buffer, pH 7.5, was applied over 6 hours at 0.15ml/minute and 0.5ml fractions were collected. The fractions were analysed for A-chain by SDS-PAGE and Western blotting. The fractions containing A-chain were pooled and dialysed against PBS.

2.20 ASSAY FOR *IN VITRO* ACTIVITY OF RIPs

2.20.1 Salt-washing ribosomes

To remove bound elongation factors and aminoacyl-tRNAs, ribosomes were washed in 0.5M KCl (Blobel, 1971). Ribosomes were incubated at a final concentration of 2mg/ml in 50mM Tris-HCl, pH 7.5, 0.5M KCl, 5mM $MgCl_2$ for 1 hour on ice. The ribosomes were pelleted at 108,000g, at 2°C for 40 minutes. The supernatant was removed and the ribosomes were washed twice in 1 X TEP buffer (3.6mM Tris, 3mM NaH_2PO_4, 0.2mM EDTA). The washed ribosomes were resuspended at 1μg/μl in 1 X TEP buffer.

2.20.2 *In vitro* assay

Incubation with RIP and aniline treatment

Aliquots containing appropriate dilutions of abrin A-chain (or PAP or ricin A-chain as a control) in PBS were incubated with 10μg of salt washed ribosomes in a 30μl reaction volume for 2 minutes at 30°C. The reaction was stopped by the addition of 60μl of water and 10μl of 10% (mass/v) SDS. The sample was extracted with phenol three times and the RNA was recovered by ethanol precipitation. The pellet was washed twice with 70% ethanol, dried under vacuum and then resuspended in water at 1μg/μl. A 2μl volume was incubated with 20μl of aniline/acetic acid, pH 4.5 at 60°C for 2 minutes, then placed on ice. The rRNA was recovered by ethanol precipitation, and the washed pellet was resuspended in 20μl 60% (v/v) formamide, pH 6.8, 0.1 X TEP buffer.

Gel analysis of RNA

A gel containing 1% agarose in 50% (v/v) formamide, 0.1 X TEP buffer was cast and assembled in a horizontal gel apparatus. The reservoir tanks were filled with 0.1 X TEP buffer to the upper level of the gel. The samples were heated to 65°C for 5 minutes, then quenched on ice. One fifth volume

of 50% (v/v) glycerol, 0.01% (mass/v) bromophenol blue was added and the samples were loaded. Electrophoresis was performed at 20mA until the dye front reached two thirds the length of the gel. The RNA was visualised by staining the gel in 1 litre of water containing 0.01mg/ml ethidium bromide with gentle agitation for 30 minutes. The gel was destained in deionised water for 3 hours with several changes of water and then photographed.

2.21 *IN VITRO* TRANSCRIPTION AND TRANSLATION

2.21.1 *In vitro* transcription

The construction of the vector for transcription *in vitro* is described in the results section (3.11.1).

The recombinant transcription vector was linearised using *EcoRI*. The DNA was recovered by ethanol precipitation and resuspended in water at 1mg/ml. The linear DNA templates (2μg) were transcribed in 40mM HEPES, pH 7.5, 6mM magnesium acetate, 2mM spermidine, 100μg/ml BSA, 10mM DTT, 0.5mM each UTP, ATP, CTP, 0.05mM GTP, 0.5 μCi [^{32}P]-UTP, 50 units RNAsin, 0.5mM $7me_5GpppG_5OH$ with 20 units SP6 polymerase at 37^{o}C. After 30 minutes dGTP was added to 3mM and the reaction mix was incubated for a further 30 minutes. An aliquot was removed and the percentage incorporation of radiolabel was determined. The RNA was extracted with phenol and recovered by ethanol precipitation. The pellet was taken up in water at 100μg/ml.

2.21.2 Rabbit reticulocyte lysate *in vitro* translation system

The RNA template was heated to 67^{o}C for 10 minutes then placed on ice prior to translation. A 2μl volume of RNA was incubated in 35μl nuclease treated reticulocyte lysate (Promega), 50 units RNAsin, 1μl 1mM L-aminoacid mixture minus

methionine and 4µl [^{35}S]-methionine (60µCi) in a 50µl reaction volume at 30^{o}C for 1 hour. The reaction was stopped by the addition of 10X SDS-PAGE loading buffer. The products of translation were analysed by SDS-PAGE and autoradiography.

2.22 TRANSLATION IN *X. LAEVIS* OOCYTES

2.22.1 Oocyte injection and homogenisation

A 30nl volume of *in vitro* transcribed mRNA at 1µg/µl was injected per oocyte and 25 oocytes were injected per mRNA species. Each set of 25 oocytes were incubated at 14^{o}C in 200µl Barth's saline supplemented with 10mg/ml penicillin and 10mg/ml streptomycin plus 50µCi [^{35}S]-methionine overnight.

The media was removed using a Pasteur pipette and the oocytes were washed twice in 200µl of Barth's saline. The washed oocytes were placed in 1ml oocyte homogenisation buffer (OHB) which consisted of 20mM Tris-HCl, pH 7.6, 0.1M NaCl, 1% (v/v) Triton X-100 and 1mM PMSF and were homogenised in a ground glass homogeniser. The sample was transferred to a microfuge tube and pelleted for 5 minutes to separate out the lipid, aqueous and particulate fractions. The aqueous fraction was recovered to a fresh tube.

Determination of Solubility

To determine the solubility of the B-chain a 40µl volume of the aqueous phase (equivalent to one oocyte) was pelleted at 100,000g at 4^{o}C for 1 hour. The supernatant was recovered to a fresh tube and the pellet was resuspended in 200µl of OHB and the centrifugation was repeated. The supernatant was recovered and pooled and the pellet was resuspended in 500µl OHB. The samples were immunoprecipitated (see below) and analysed by SDS-PAGE and autoradiography.

Immunoprecipitation

Prior to immunoprecipitation all sample volumes were made up to 500ul with OHB. To each 500µl sample, an equal volume of immunoprecipitation buffer (IPB) consisting of 0.1M Tris-HCl, pH 7.6, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (mass/v) SDS, 10% (mass/v) deoxycholate free acid, (adjusted to pH 8.2 with 5M NaOH), was added. The sample was mixed gently then 5-10µl of the required anti-sera or antibody, and 40µl of protein A-Sepharose were added. The samples were rotated gently for 2 hours at room temperature. The Sepharose beads were pelleted for 10 seconds in a microfuge and the supernatant was discarded. The beads were washed 4 times in IPB and then suspended in 40µl of SDS-PAGE loading buffer. The samples were boiled for 2 minutes, quenched on ice then pelleted briefly. The supernatant was analysed by SDS-PAGE and autoradiography.

2.22.2 Assay for lectin activity

Immobilised lactose column

A 1cm by 10cm column was packed with 0.5ml lactose immobilised on aminoethylated polyacrylamide beads. The column was equilibrated with 5ml OHB. A 200µl volume of aqueous homogenate was made up to 1ml with OHB and passed down the column. The eluted sample was then repassed down the column twice. The column was washed with 3ml OHB and 1ml fractions were collected. Any bound protein was eluted with 3ml OHB containing 50mM lactose and 1ml fractions were collected. The procedure was carried out at 4°C. A 500µl volume of each fraction was immunoprecipitated and analysed by SDS-PAGE and autoradiography.

Asialofetuin Plate assay

To the wells of a 96 well microtitre plate, 300µl of 1mg/ml asialofetuin in 0.1M $NaHCO_3$, pH 9.2, was added and the plate was stored at 4°C for at least 6 hours to allow the asialofetuin to bind. The asialofetuin was removed and each

Immunoprecipitation

Prior to immunoprecipitation all sample volumes were made up to 500ul with OHB. To each 500µl sample, an equal volume of immunoprecipitation buffer (IPB) consisting of 0.1M Tris-HCl, pH 7.6, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (mass/v) SDS, 10% (mass/v) deoxycholate free acid, (adjusted to pH 8.2 with 5M NaOH), was added. The sample was mixed gently then 5-10µl of the required anti-sera or antibody, and 40µl of protein A-Sepharose were added. The samples were rotated gently for 2 hours at room temperature. The Sepharose beads were pelleted for 10 seconds in a microfuge and the supernatant was discarded. The beads were washed 4 times in IPB and then suspended in 40µl of SDS-PAGE loading buffer. The samples were boiled for 2 minutes, quenched on ice then pelleted briefly. The supernatant was analysed by SDS-PAGE and autoradiography.

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Asialofetuin Plate assay

To the wells of a 96 well microtitre plate, 300µl of 1mg/ml asialofetuin in 0.1M $NaHCO_3$, pH 9.2, was added and the plate was stored at 4°C for at least 6 hours to allow the asialofetuin to bind. The asialofetuin was removed and each

well was filled with blocking solution consisting of PBS, 0.05% (mass/v) BSA and 0.05% (v/v) Tween-20. The plate was stored at 4°C overnight. Just prior to the assay the blocking solution was removed.

A 200µl volume of oocyte homogenate was added to each well and incubated at room temperature for 2 hours. The sample was removed and the wells were washed three times with PBS. A 1 in 50 dilution of sheep anti-ricin B-chain antibody, which had previously been shown to cross react with the abrin B-chain, in blocking solution was made and 250µl was then added to each well. The plate was incubated at room temperature for 2 hours. The antibody was removed, the wells were washed three times with PBS then 250µl of a 1 in 250 dilution of [^{125}I]-Protein G (kindly provided by Dr R. Wales, University of Warwick) in blocking solution was added. The plate was incubated for 2 hours at room temperature. The wells were washed four times with PBS before counting in a Gamma counter.

To obtain a standard curve for the assay, purified ricin B-chain (at 5mg/ml) was diluted accordingly and used as a positive control in place of the oocyte homogenate. Each control and test sample was assayed in triplicate.

2.22.3 Reassociation of B-chain with recombinant A-chain

At least 2µg i.e. a 50 fold excess, of either recombinant abrin A-chain or recombinant ricin A-chain were added to 200µl of homogenate and the volume was made up to 500µl with OHB. The samples were rotated for at least 4 hours at 4°C. To determine any reassociation with A-chain the samples were divided and immunoprecipitated with anti-ricin A-chain antibodies. The immunoprecipitated protein was analysed by reducing and non-reducing SDS-PAGE and autoradiography.

3.RESULTS AND DISCUSSION

3.1 PREPARATION AND ANALYSIS OF GENOMIC DNA

3.1.1 Preparation of genomic *A. precatorius* DNA

Leaves from *A. precatorius* were harvested in southern Florida onto solid CO_2 for shipment and were stored at -70°C. Approximately 3.5mg of high molecular mass DNA was obtained from 10g of frozen *A. precatorius* leaf tissue. This yield was considerably lower than the yield of DNA obtained from frozen *M. charantia* tissue using the same method. Several factors could have influenced the yield of DNA including the DNA content, the number of cells/g tissue, water content and the growth state of the leaves. *A. precatorius* leaves are small and of low water content which made the grinding difficult and inefficient. The method of DNA extraction used, modified from a procedure described by Taylor and Powell (1983), removed the majority of contaminants such as polysaccharides, polyphenols and pigments which can co-purify with the DNA and interfere with cloning efficiency for plant DNA. Generally the efficiency of cloning of plant DNA is reduced by one- to three-fold lower than that of bacterial and animal DNA

All the type II ribosome-inactivating proteins that have been sequenced to date lack introns (Halling *et al.*, 1985) and the plant lectin genes sequenced to date lack introns. The soybean lectin gene (Vodkin, 1983) and *Phaseolus vulgaris* lectin genes (Hoffman, 1984 and Hoffman and Donaldson, 1985) all lack introns. In addition certain other plant genes lack introns which include all the genes which have been sequenced for the maize storage protein (zein), (Pederson *et al.*, 1982 and Hu *et al.*, 1982). It was therefore highly probable that abrin-related genes would also lack introns.

The genomic DNA cloning approach overcame the problem of obtaining mRNA from developing seeds. The temporal expression of ricin in the developing seed has been studied (Tregear,

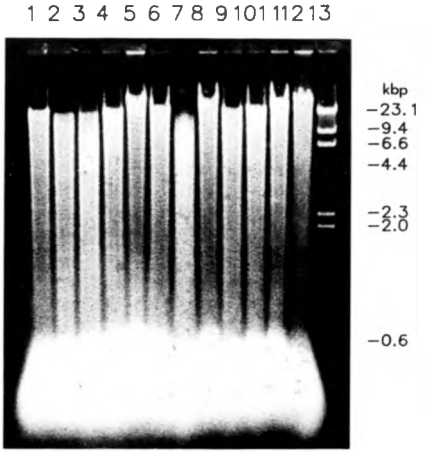
therefore suggest that abrin-related mRNAs would also be relatively abundant during late seed maturation. However, obtaining developing seeds would have been difficult and genomic DNA can be isolated from the more convenient leaf tissue.

3.1.2 Digestion of genomic DNA

Figure 7 shows genomic *A. precatorius* DNA digested to completion with a variety of restriction enzymes. The restriction enzymes chosen were selected for the presence of their recognition sites in the cloning site of the lambda phage vectors Lambda ZAPII and Lambda DASHII. Lane 12 shows uncut DNA which is approximately 50kb in size. Sizing of the uncut DNA, on a sucrose gradient for example, to remove the smaller DNA fragments was omitted to avoid further handling of the high molecular mass DNA. The planned method, of generating a probe by PCR and of generating lambda phage libraries using a complete digest of the genomic DNA, circumvented the need to remove the smaller fragments.

Digestion of the genomic DNA with each of the restriction endonucleases used led to loss of the very high molecular mass DNA showing that the method employed for isolation of genomic DNA yielded DNA which was pure enough for digestion by the majority of restriction endonucleases. Digestion with *NotI* (lane 5), which recognises an octanucleotide sequence, generated significantly less of a smear. There are bands of DNA visible against the background smear which is indicative of repetitive DNA.

Figure 7 *Restriction endonuclease analysis of genomic A. precatorius DNA* Approximately 10μg of *A. precatorius* DNA was digested with various restriction endonucleases. Lanes 1-11, *BamHI*, *EcoRI*, *HindIII*, *NcoI*, *NotI*, *PstI*, *SacI*, *SalI*, *SpeI*, *XbaI* and *XhoI* respectively; lane 12, uncut DNA; lane 13, lambda/*HindIII* DNA markers



3.2 GENERATION OF AN ABRIN-SPECIFIC PROBE

3.2.1 Primer design for polymerase chain reaction

The complete amino acid sequence of abrin C A-chain was used to design primers for PCR on genomic DNA. The protein sequence data were converted into degenerate nucleic acid coding sequence by reverse translation using the universal genetic code. Figure 8 and 9 show the complete sequence of abrin C A-chain converted into mRNA and cDNA respectively. An N-terminal region and an internal region, each approximately 12 amino acids long, were selected for primer design.

Previous experiments carried out using shorter primers (25 mers) on *M. charantia* genomic DNA, in PCR, were unsuccesful due to a high background of product generated from non-specific priming. Longer primers were therefore designed (30-35 mers) to reduce non-specific binding. Figure 10 shows the design of the primers that were synthesised. To reduce the overall complexity of each primer pool, deoxyinosines were included at certain positions where all four nucleotides were possible. Inosine is occasionally found in the first position of tRNA anticodons (Crick, 1966) and is known to form base pairs with A, C and U. Ohtsukan *et al.* (1985) have shown that deoxyinosine residues in an oligonucleotide sequence do not seriously disturb DNA duplex formation nor destabilise the duplex. Deoxyinosine substitutions were distal to the 3' end to ensure specific binding and each primer ended with a GC pair to stabilise the 3' end for primer extension during PCR. The inclusion of deoxyinosines reduced the number of primers in pool ABR-N from 3,145,728 to 3,078 and in primer pool ABR-INT from 104,704 to 781. The codon usage in plants does not show a significant bias (for review see Brown, 1990) therefore the number of isomers could not be reduced further by omitting particular codons.

Figure 8 *Reverse translation of the primary sequence of abrin C A-chain into mRNA* The primary sequence is numbered according to the published sequence (Funatsu *et al.*, 1988). The mRNA sequence is shown below where N represents A, C, G or U, R represents A or G, Y represents U or C and H represents A, C or U. The domain selected for primer design is shown underlined

```
 1- Glu Asp Arg Pro Ile Lys Phe Ser Thr Glu Gly Ala Thr Ser Gln
    GAR GAY CGN CCN AUN AAR UUY UCN ACN GAR GGN GCN ACN UCN CAR
            AGR                 AGY                     AGY

16- Ser Tyr Lys Gln Phe Ile Glu Ala Leu Arg Glu Arg Leu Arg Gly
    UCN UAY AAR CAR UUY AUN GAR GCN UUR CGN GAR CGN UUR CGN GGN
    AGY                         CUN AGR     AGR CUN AGR

31- Gly Leu Ile His Asp Ile Pro Val Leu Pro Asp Pro Thr Thr Leu
    GGN UUR AUN CAY GAY AUN CCN GUN UUR CCN GAY CCN ACN ACN UUR
            CUN                 CUN                     CUN

46- Gln Glu Arg Asn Arg Tyr Ile Thr Val Glu Leu Ser Asn Ser Asp
    CAR GAR CGN AAY CGN UAY AUN ACN GUN GAR UUR UCN AAY UCN GAY
            AGR     AGR                 CUN AGY         AGY

61- Thr Glu Ser Ile Glu Val Gly Ile Asp Val Thr Asn Ala Tyr Val
    ACN GAR UCN AUN GAR GUN GGN AUN GAY GUN ACN AAY GCN UAY GUN
            AGY

76- Val Ala Tyr Arg Ala Gly Thr Gln Ser Tyr Phe Leu Arg Asp Ala
    GUN GCN UAY CGN GCN GGN ACN CAR UCN UAY UUY UUR CGN GAY GCN
                AGR                 AGY         CUN AGR

91- Pro Ser Ser Ala Ser Asp Tyr Leu Phe Thr Gly Thr Asp Gln His
    CCN UCN UCN GCN UCN GAY UAY UUR UUY ACN GGN ACN GAY CAR CAY
    AGY AGY     AGY             CUN

106- Ser Leu Pro Phe Tyr Gly Thr Tyr Gly Asp Leu Glu Arg Trp Ala
     UCN UUR CCN UUY UAY GGN ACN UAY GGN GAY UUR GAR CGN UGG GCN
     AGY CUN                         CUN         AGR

121- His Gln Ser Arg Gln Gln Ile Pro Leu Gly Leu Gln Ala Leu Thr
     CAY CAR UCN CGN CAR CAR AUN CCN UUR GGN UUR CAR GCN UUR ACN
             AGY AGR                 CUN     CUN             CUN

136- His Gly Ile Ser Phe Phe Arg Ser Gly Gly Asn Asp Asn Glu Glu
     CAY GGN AUN UCN UUY UUY CGN UCN GGN GGN AAY GAY AAY GAR GAR
                 AGY         AGR AGY

151- Lys Ala Arg Thr Leu Ile Val Ile Ile Gln Met Val Ala Glu Ala
     AAR GCN CGN ACN UUR AUN GUN AUN AUN CAR AUG GUN GCN GAR GCN
             AGR     CUN

166- Ala Arg Phe Arg Tyr Ile Ser Asn Arg Val Arg Val Ser Ile Gln
     GCN CGN UUY CGN UAY AUN UCN AAY CGN GUN CGN GUN UCN AUN CAR
         AGR             AGY     AGR     AGR         AGY

181- Thr Gly Thr Ala Phe Gln Pro Asp Ala Ala Met Ile Ser Leu Glu
     ACN GGN ACN GCN UUY CAR CCN GAY GCN GCN AUG AUN UCN UUR GAR
                                                 AGY CUN

196- Asn Asn Trp Asp Asn Leu Arg Gly Val Gln Glu Ser Val Gln Asp
     AAY AAY UGG GAY AAY UUR CGN GGN GUN CAR GAR UCN GUN CAR GAY
                 CUN AGR                     AGY

211- Thr Phe Pro Asn Gln Val Thr Leu Thr Asn Ile Arg Asn Glu Pro
     ACN UUY CCN AAY CAR GUN ACN UUR ACN AAY AUN CGN AAY GAR CCN
                     CUN                     AGR

226- Val Ile Val Asp Ser Leu Ser His Pro Thr Val Ala Val Leu Ala
     GUN AUN GUN GAY UCN UUR UCN CAY CCN ACN GUN GCN GUN CUN GCN
                 AGY CUN AGY                             CUN

241- Leu Met Leu Phe Val Cys Asn Pro Pro Asn
     UUR AUG UUR UUY GUN UGY AAY CCN CCN AAY
     CUN     CUN
```

Figure 9 *Reverse translation of the primary sequence of abrin C A-chain into complementary DNA* The primary sequence is numbered according to the published sequence (Funatsu *et al.*, 1988). The complementary DNA sequence is shown below where N represents A, C, G or T, R represents A or G, Y represents T or C and H represents T, C or A. The region selected for primer design is shown underlined

Figure 10 *Degenerate oligonucleotide primer design* The N-terminus and internal regions of abrin C A-chain selected for primer design are numbered according to the published sequence (Funatsu *et al.*, 1988) and the primer pools that were synthesised are shown below. The orientation of the primers is indicated with primer pool ABR-INT representing complementary DNA. Nucleotides included at each position are listed vertically and positions chosen for deoxyinosine substitutions (I) are shown

Primer pool ABR-N

```
     11                                                    23
     Gly Ala Thr Ser Gln Ser Tyr Lys Gln Phe Ile Glu Ala
5'-  GGG GCG ACI III CAA III TAT AAA CAA TTT ATT GAA GC -3'
       A   A           G       C   G   G   C   C   G
       T   T                                   A
       C   C
```

Primer pool ABR-INT

```
        190                                                200
        Ala Met Ile Ser Leu Glu Asn Asn Trp Asp Asn
3'-     CGG TAC TAA III IAI CTT TTA TTA ACC CTA TT -5'
          A   A   T               A   G   G       G
          T       G
          C
```

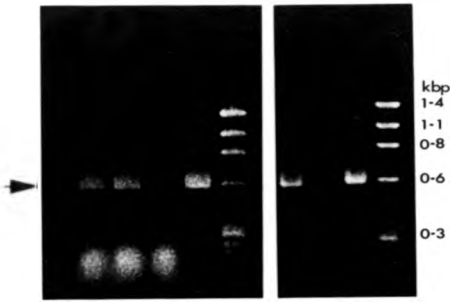
3.2.2 Polymerase chain reaction

The primer pools ABR-N and ABR-INT were used in PCR on genomic *A. precatorius* DNA template. Figure 11A shows analysis of the PCR products by agarose gel electrophoresis using different temperatures for annealing and denaturation. Lane 5 shows the major product of 570bp using the standard conditions for PCR of denaturation at 92°C, annealing at 50°C and extension at 72°C. A 570bp fragment was the expected size based on placement of the primers on the derived coding sequence of abrin C A-chain. The major effect on the amount of PCR product was temperature of annealing: at 40°C (lane 4) and at 58°C (lane 8) a 570bp product was not detected on agarose gels stained with ethidium bromide, whereas a 570bp product was clearly visible at annealing temperatures of 42°C (lane 3) and 48°C (lane 2). The amount of 570bp product was not improved by increasing the denaturing temperature from 94°C to 98°C for the first 5 cycles (lane 7) indicating that the genomic DNA was fully denatured at the lower temperature of 92°C, for 1 minute.

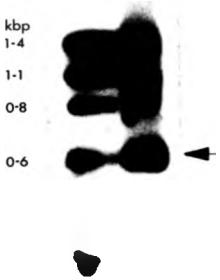
When the primers were labelled prior to PCR with γ-[^{32}P] the 570bp fragment was radiolabelled as determined by autoradiography of the PCR products on agarose gel shown in Figure 11B. The smear of radiolabelled product larger than 570bp is due to the long form PCR products derived from primer extension on the original genomic DNA template. It is also a possibility that the primers may be annealing to separate and distinct genes on the genomic DNA template, generating a PCR product extending from the 5' end of an upstream abrin-related gene, across any intervening sequence, to the internal domain of a second downstream abrin-related gene. Two faint bands of approximately 1.35kbp and 1.0kbp which appear as more of a smear are too small to be due to annealing to distinct separate genes.

Figure 11 *Analysis of the products of PCR using primers ABR-N and ABR-INT on genomic DNA template* A standard PCR was carried out as described (section 2.7.1) and 5% of the end-reaction volume was analysed by agarose gel electrophoresis. The effect of the temperature of annealing and of denaturation was determined empirically (A): lane 1, no primers or template in PCR; lanes 3-5, annealing at 42°C, 40°C and 50°C respectively; lanes 6 and 10, ΦX174/*HaeII* (1.4kb, 1.1kb, 0.8kb, 0.6kb, 0.3kb, 0.28kb, 0.23kb, 0.2kb and 0.12kb); lane 7, denaturation at 94°C; lanes 8 and 9, annealing at 58°C and 50°C respectively. A standard PCR was carried out using α-^{32}P-radiolabelled primers and the products were analysed on agarose gel and visualised by autoradiography (B): lane 1, ΦX174/*HaeII* DNA markers; lane 2, radiolabelled PCR products. The arrow indicates the 570bp product

A) 1 2 3 4 5 6 7 8 9 10



B) 1 2



3.2.3 Analysis of 570bp fragment

The 570bp fragment was sequenced directly as described (section 2.8.2). The derived amino acid sequence from the determined nucleic acid sequence was closely related to abrin C A-chain confirming that the 570bp fragment was abrin-related. To produce large amounts of the fragment for radiolabelling for use as a probe, the 570bp fragment was subcloned: PCR was repeated using the linker extended primers ABR-NX and ABR-INTX shown in Figure 12, the fragment was isolated and subcloned into the Bluescript vector. The original primer pool design was used but each primer was extended at the 5' end with a linker which would generate the recognition sites for *XhoI* at the 5' end and *SacII* at the 3' end of the PCR product. These two restriction endonucleases were selected since they cut genomic *A. precatorius* DNA less frequently than the other restriction endonucleases analysed. The primer sequence derived from abrin C A-chain was shortened to 6 amino acids to circumvent the use of deoxyinosine substitutions and ensure specific priming. The primers ABR-NX and ABR-INTX were used in a standard PCR reaction on genomic DNA template, the 570bp fragment was recovered from agarose gel, digested sequentially with *XhoI* and *SacII* then subcloned into pBluescript SK+. This allowed the insert to be fully sequenced and the sequence obtained is shown in Figure 13. The sequence obtained was identical to and extended the sequence determined from direct sequencing of the 570bp PCR product. The abrin-related probe was removed from the vector by digestion and was recovered from agarose gel prior to radiolabelling by random priming.

Figure 12 *Linker-extended degenerate oligonucleotides, ABR-NX and ABR-INTX* The coding sequence of abrin C A-chain is numbered according to the published sequence (Funatsu *et al.*, 1988) and the primer pools that were synthesised are shown below. The orientation of the primers is indicated with primer pool ABR-INTX representing complementary DNA. Nucleotides included at each position are listed vertically. The linker regions, to generate restriction endonuclease recognition sites, are underlined

Primer pool ABR-NX

```
                  17                                    23
                  Tyr Lys Gln Phe Ile Glu Ala
5'- GG CTC GAG    TAT AAA CAA TTT ATT GAA GC -3'
      XhoI         C   G   G   C   C   G
                                   A
```

Primer pool ABR-INTX

```
   194                          200
   Leu Glu Asn Asn Trp Asp Asn
3'- AT CTT TTA TTA ACC CTA TTG GCG CCG G -5'
     C   C   G   G       G     SacII
```

Figure 13 *Nucleotide sequence of the 570bp abrin-related probe*
The nucleic acid sequence is numbered starting with the first nucleotide deduced within the 570bp fragment. One long continuous open reading frame was obtained on translation and this is shown below. The homologous region from abrin C A-chain is shown in italics. Amino acid changes between the abrin-related probe and abrin C are highlighted

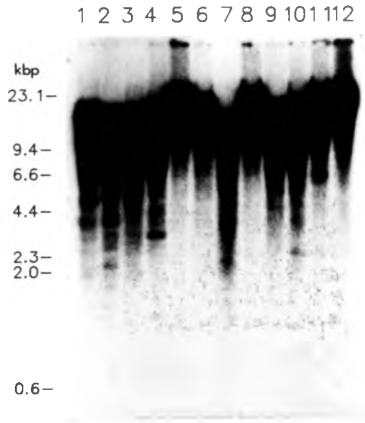
```
   1 CTT CGA CAG AGA CTA ACA GGT GGG CTG ATA CAT GGC ATA CCT  42
     Leu Arg Gln Arg Leu Thr Gly Gly Leu Ile His Gly Ile Pro
     Leu Arg Glu Arg Leu Arg Gly Gly Leu Ile His Asp Ile Pro

  43 GTG CTT CCA GAT CCA ACA ACA TTG CAA GAA AGA AAT CGA TAT  84
     Val Leu Pro Asp Pro Thr Thr Leu Gln Glu Arg Asn Arg Tyr
     Val Leu Pro Asp Pro Thr Thr Leu Gln Glu Arg Asn Arg Tyr

  85 ATT TCA GTC GAA CTC TCA AAC TCG GAT ACG GAA TCT ATC GAA 126
     Ile Ser Val Glu Leu Ser Asn Ser Asp Thr Glu Ser Ile Glu
     Ile Thr Val Glu Leu Ser Asn Ser Asp Thr Glu Ser Ile Glu

 127 GCA GGT ATC GAT GTG AGT AAT GCA TAT GTT GTA GCA TAC CGA 168
     Ala Gly Ile Asp Val Ser Asn Ala Tyr Val Val Ala Tyr Arg
     Val Gly Ile Asp Val Thr Asn Ala Tyr Val Val Ala Tyr Arg

 169 GCA GGA AAT CGG TCT TAT TTC CTC CGT GAT GCC CCA ACA GCT 210
     Ala Gly Asn Arg Ser Tyr Phe Leu Arg Asp Ala Pro Thr Ala
     Ala Gly Thr Gln Ser Tyr Phe Leu Arg Asp Ala Pro Ser Ser

 211 GCA TCT AGG TAC CTT TTC ACT GGC ACG CAG CAG TAC CTC GCT 252
     Ala Ser Arg Tyr Leu Phe Thr Gly Thr Gln Gln Tyr Leu Ala
     Ala Ser Asp Tyr Leu Phe Thr Gly Thr Asp Gln His Ser Leu

 253 TCG TTT AAT GGT AGT TAT ATT GAT CTA GAG AGA TTG GCT CGT 294
     Ser Phe Asn Gly Ser Tyr Ile Asp Leu Glu Arg Leu Ala Arg
     Pro Phe Tyr Gly Thr Tyr Gly Asp Leu Glu Arg Trp Ala His

 295 CAG ACA AGA CAG CAA ATA CCC CTG GGG TTA CAG GCC TTG AGA 336
     Gln Thr Arg Gln Gln Ile Pro Leu Gly Leu Gln Ala Leu Arg
     Gln Ser Arg Gln Gln Ile Pro Leu Gly Leu Gln Ala Leu Thr

 337 CAT GCA ATA TCG TTT TTC TCG AGA GGT GGA CTG ATG ATC GAA 378
     His Ala Ile Ser Phe Phe Ser Arg Gly Gly Leu MET Ile Glu
     His Gly Ile Ser Phe Phe Arg Ser Gly Gly Asn Asp Asn Glu

 379 GAA ATA GCG CGT ACC CTG ATC GTG ATA ATC CAA ATG GCT TCA 420
     Glu Ile Ala Arg Thr Leu Ile Val Ile Ile Gln MET Ala Ser
     Glu Lys Ala Arg Thr Leu Ile Val Ile Ile Gln Met Val Ala

 421 GAA GCA GCT CGA TAC AGG TTC ATA TCA TAC CGG GTT GGT GTC 462
     Glu Ala Ala Arg Tyr Arg Phe Ile Ser Tyr Arg Val Gly Val
     Glu Ala Ala Arg Phe Arg Tyr Ile Ser Asn Arg Val Arg Val

 463 AGC ATC CGA ACT GGT ACG GCG TTT CAA CCT GAT GCT GCA ATG 504
     Ser Ile Arg Thr Gly Thr Ala Phe Gln Pro Asp Ala Ala MET
     Ser Ile Gln Thr Gly Thr Ala Phe Gln Pro Asp Ala Ala Met
```

Several distinct isoforms of abrin have been described and therefore the 570bp fragment may have been derived from several of the abrin genes. If this had been the case the sequence obtained from direct sequencing of the PCR product would expected to have been mixed. Only a single sequence was detected, therefore, although subcloning would select only one DNA molecule from the many within each pool from PCR, this cloned fragment would be representative of the most abundant amplified DNA. This was confirmed by the sequencing of the cloned fragment which was 100% identical with the sequence obtained from the entire pool of 570bp fragments.

3.3 SOUTHERN BLOT ANALYSIS

A Southern blot of genomic *A. precatorius* DNA digested with a variety of restriction endonucleases and probed with the abrin-related 570bp fragment is shown in Figure 14. Lane 12 confirms that a proportion of uncut DNA is of high molecular mass. It is apparent that digestion with *NotI* (lane 5), *PstI* (lane 6), *SalI* (lane 8) and *XhoI* (lane 11) generated positive hybridising bands of relatively high molecular mass and would be unsuitable for use for library generation in standard lambda phage libraries. Digestion with *SalI* (lane 7) gave a smear of positive hybridising DNA but no distinct bands were visible. Of the remainder, digestion with *EcoRI* (lane 2) gave a good range of positive hybridising bands from 0.5kbp to 10kbp and *EcoRI* can be used to linearise both lambda ZAPII and lambda DASHII.

Figure 14 *Southern blot analysis of A. precatorius genomic DNA* Genomic DNA (10μg) was digested with a variety of restriction endonucleases, electrophoretically separated on 0.8% agarose/TAE gel and transferred to nitrocellulose. Radiolabelled abrin-related 570bp fragment was used as probe. Lanes 1-11, DNA digested with *BamHI*, *EcoRI*, *HindIII*, *NcoI*, *NotI*, *PstI*, *SacI*, *SalI*, *SpeI*, *XbaI and XhoI* respectively; lane 12, uncut DNA



The results of the Southern blot indicate that the genome size of *A. precatorius* is compatible with standard lambda phage library banks: as a crude estimate for a genome size of 3 x 10^{10}bp, with approximately 1 x 10^{5} genes each on average 1kb in size, a single copy gene will be represented by 10pg out of 10μg genomic DNA which is detectable by Southern blot. The presence of several positive hybridising bands in several tracks indicates that there are at least five abrin-related genes which was expected from the number of isoforms of abrin described previously (Lin *et al.*, 1981).

3.4 GENERATION OF LAMBDA LIBRARY BANKS

3.4.1 Amplified/Gigapack Gold packaged libraries

Initially all library banks were generated using Gigapack Gold (Stratagene) packaging extracts and the host strain XLI-Blue. The packaged library was subjected to a single round of amplification, before plating out, to generate a high titre library for screening. Several million plaques were screened from both a lambda ZAPII and a lambda DASHII library and no positive-hybridising plaques were obtained after secondary screening.

The Southern blot indicated that there were several abrin-related genes, and that the genome size was compatible with standard lambda phage libraries so lack of any positive-hybridising plaques was unexpected. Although the efficiency of cloning of plant DNA is generally lower than for animal or bacterial DNA due to contamination of the DNA, in particular with polysaccharides, the plant DNA here was sufficiently pure to allow digestion by a wide variety of restriction endonucleases.

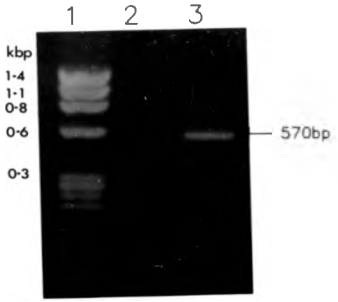
A possible cause may have been the round of amplification used since not all recombinant phage will amplify to the same titre and this may have led to under-representation of

sequences of interest. However, in addition to this, methylation-dependent restriction systems may be relevant. Plant DNA is often highly methylated: up to 50% of all cytosines may be methylated including 80% of CG and $C^{A}/_{T}G$ sequences (van Etten *et al.*, 1988 and Blumental, 1989). The modified cytosine restriction (Mcr) system (Vanyushii and Kernos, 1988) may therefore be of particular importance: on introduction to *mcr*$^{+}$ *E.coli* the more heavily methylated DNA will be preferentially restricted.

3.4.2 Packaging with Mcr^{-} extracts

A new commercially available packaging extract became available called Gigapack II Gold which was prepared from *mcr*$^{-}$ *E. coli*. To determine if Mcr restriction and amplification were leading to loss of representation of sequences of interest within the libraries, PCR was used to confirm the presence of abrin-related sequences. PCR was carried out using primer pools ABR-N and ABR-INT on 1µl of a 1 to 100 dilution in water of either a Gigapack Gold packaged and amplified library, or a Gigapack II Gold packaged library without amplification, in a standard reaction. Figure 15 shows analysis of the PCR products on agarose gel and a 570bp band is visible only when the template was from Gigapack II Gold packaged DNA and prior to amplification (lane 3). A Southern blot of this gel probed with the radiolabelled 570bp fragment indicated that a very faint band was present in the Gigapack Gold packaged and amplified library (lane 2) however since the titre of this library was several fold higher than the Gigapack II Gold packaged library it was clear that the modified cytosine restriction system and/or the amplification step was leading to a significant under-representation of abrin-related sequences.

Figure 15 *Analysis of packaged libraries for sequences of interest by PCR* A 1µl aliquot of a 1 in 100 dilution of packaged library extract was used as template in PCR using primers ABR-N and ABR-INT. 5% of the end-reaction mix was analysed by agarose gel electrophoresis. Lane 1, øX174/*HaeII* DNA markers; lane 2, Gigapack Gold packaged and amplified library extract; lane 3, Gigapack Gold II packaged, unamplified library extract



Libraries were generated using the Gigapack II Gold packaging extract and were plated out for filter lifts without an amplification step. Table 3 below, shows the titres, efficiency of plating and percentage recombinants determined for test ligations of genomic DNA into lambda ZAPII and lambda DASHII vectors.

Table 3 *Titres, efficiency of plating and percentage recombinants for test ligation of genomic A.precatorius DNA/EcoRI into Lambda ZAPII/EcoRI and Lambda DASHII/EcoRI vectors* The optimum titres for recombinant lambda ZAPII and lambda DASHII libraries, without amplification, were obtained from ligations containing 250ng and 500ng respectively of genomic *A. precatorius* DNA. Percentage recombinants in lambda ZAPII libraries were determined by blue/white colour selection in the presence of X-gal and IPTG. Percentage recombinants in lambda DASHII libraries were determined by sensitivity to the P2 lysogen (see section 2.9.4 for host strains)

	Lambda ZAPII	Lambda DASHII
Efficiency of plating (per μg lambda arms)	3.6×10^6	2.8×10^6
Titre (per ml)	4.2×10^6	4.8×10^5
% recombinants	99.8	99.1

3.5 SCREENING OF RECOMBINANT LIBRARIES

3.5.1 Lambda ZAPII libraries

After final stringent wash conditions in 0.1XSSC at 65°C for 20 minutes, a total of 10 positive-hybridising plaques were obtained from the lambda ZAPII library of which four gave a strong signal. The other six plaques gave weak signals under the conditions used and these six plaques were picked, a liquid lysate was prepared for each of them and the lysates were stored for later analysis. The four strongly-hybridising plaques were converted to double stranded recombinant Bluescript phagemid as described (section 2.15.4).

3.5.2 Lambda DASHII libraries

After final stringent wash conditions in 0.1XSSC at 65°C for 20 minutes a total of three positive-hybridising plaques were obtained. The degree of hybridisation was weak relative to the strongly-hybridising plaques from the lambda ZAP II library bank. The plaques were cored, a liquid lysate was prepared from each and stored for later analysis.

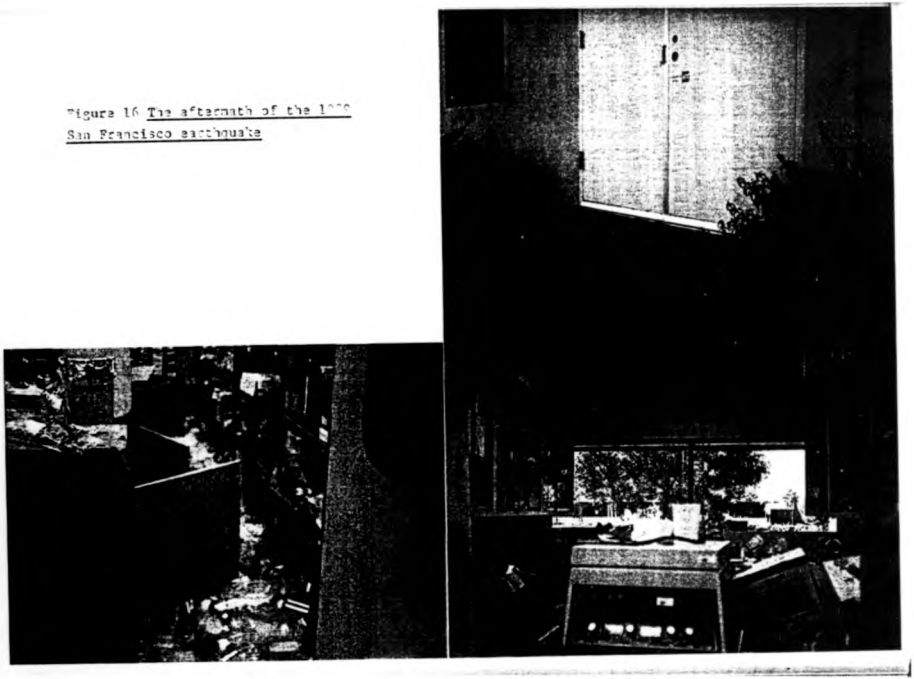
3.5.3 Summary of generation and screening of genomic libraries

PCR was successfully used to prove sequences of interest were represented within the libraries. By this method it was shown that abrin-related sequences were represented in standard lambda phage libraries only when packaging was carried out in extracts derived from *mcr*$^-$ host strains and the packaged DNA was plated out without amplification.

A total of 13 positive-hybridising plaques were obtained of which four were analysed further. The remaining 9 weakly-hybridising plaques were stored as phage lysates for later analysis. Unfortunately, these stored lysates were destroyed

during the San Francisco earthquake of October 1989 before further analysis. Figure 16 shows the cold room where the lysates were stored, after the earthquake hit.

Figure 16 The aftermath of the 1906 San Francisco earthquake



3.6 ANALYSIS OF RECOMBINANT PHAGEMIDS

3.6.1 Analysis by restriction mapping

Large scale plasmid DNA was prepared for each positive clone and analysed initially by restriction mapping. Figure 17 shows the restriction maps determined for the four clones pQK2, pQK3, pQK7 and pQK9. The three clones pQK2, pQK3 and pQK7 had identical restriction maps. The similarity expected between the isoforms of abrin-related genes would also be reflected in the similarity between restriction maps. To determine identity, the 5' and 3' ends of the inserts within these three clones were sequenced using the T3 and T7 priming sites within the vector. The sequence of several hundred base pairs at both the 5' and 3' ends showed the three clones were identical over the regions sequenced. Open reading frames were not found in these flanking regions, therefore, the 100% identity between this non-coding DNA showed that the three clones pQK2, pQK3, and pQK7 were identical and allowed selection of pQK3 as a representative for complete sequencing.

3.6.2 Nucleic acid sequencing strategy

The sequencing strategy used is shown in Figure 18. The restriction mapping of the clones pQK3 and pQK9 showed that sequencing overlapping subcloned fragments of the insert would be lengthy due to the limited number of convenient unique restriction endonuclease sites. To avoid this the inserts were sequenced inwards from the T7 and T3 priming sites of the vector and thereafter primers were generated as required. Both strands of the plasmid DNA was sequenced and all sequencing reactions were repeated with the dITP termination mixes to ensure any compressions were fully denatured.

Figure 17 *Partial restriction maps of the inserts of clones pQK2, pQK3, pQK7 and pQK9* The partial restriction maps of the inserts of (A) pQK2, pQK3 and pQK7 and (B) pQK9 are shown. The restriction endonuclease sites determined and their distance in kilobases from the 5' *EcoRI* cloning site in the vector is indicated

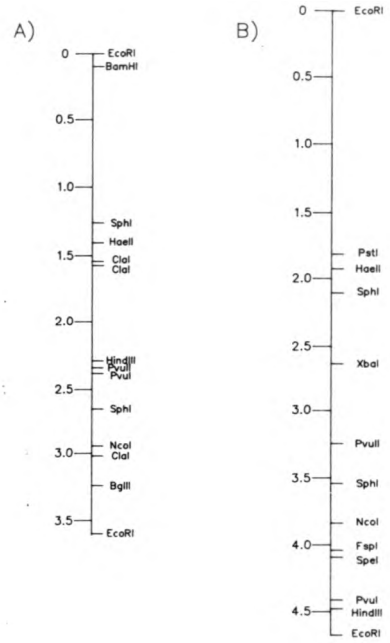
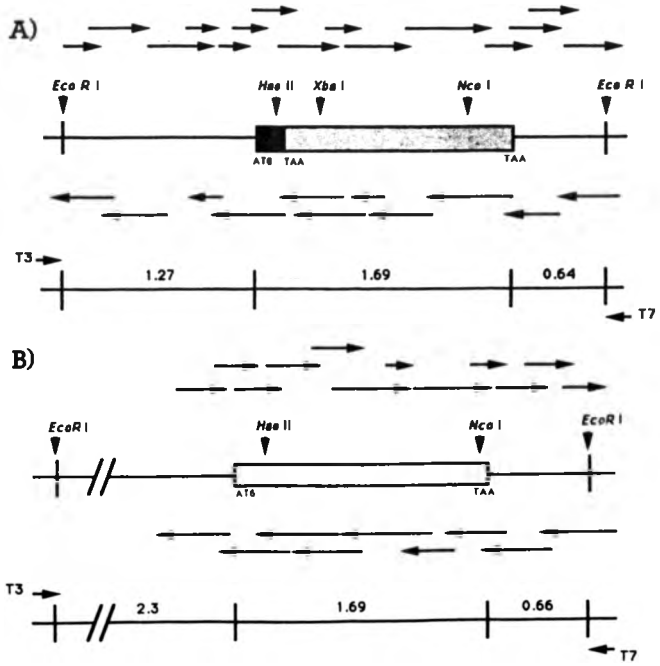


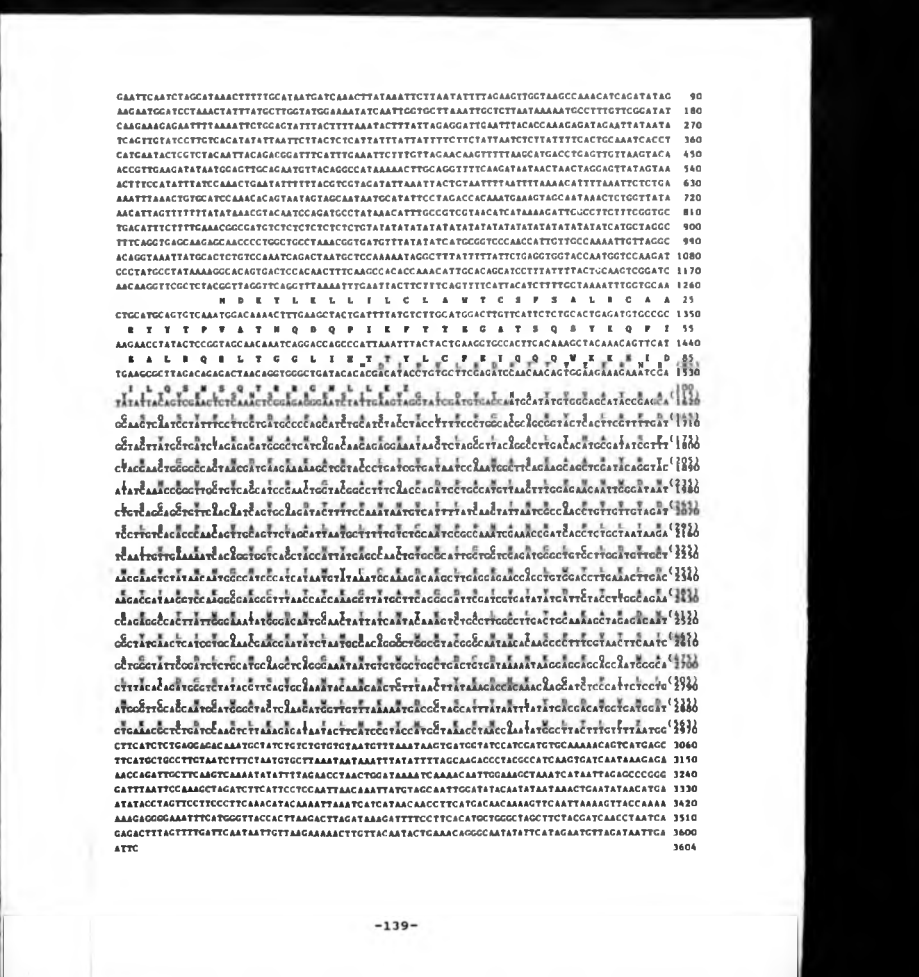
Figure 18 *DNA sequencing strategy on pQK3 and pQK9* A schematic diagram of the *EcoRI* insert in (A) pQK3 and (B) pQK9 is shown. The arrows indicate the approximate length of sequence reads from the ends of the primers used. The sequenced regions encoding open reading frames are boxed. pQK3 contains a frameshift and this is indicated. The position of relevant restriction sites are noted. A distance scale is given relative to the T3 and T7 priming sites in the vector



3.6.3 Sequence analysis of pQK3

Analysis of the sequence of the insert of pQK3 by translation into amino acid sequence (Figure 19) showed that a putative long open reading frame encoding a polypeptide of 563 amino acids was disrupted by an insertion of AC after amino acid 67. The frameshift caused by this two base insertion leads to a stop codon after amino acid 104. This two base insertion was the only frameshift within the putative long open reading frame and was only revealed by sequencing using the dI termination mixes. It is possible that this two base insertion was generated during the cloning procedure. However mutations that lead to reduction or elimination of seed polypeptides have been found in several different plant species, including gene inactivation due to translational frameshift (Goldberg, 1989) and several ricin pseudogenes have been described (Tregear, 1990).

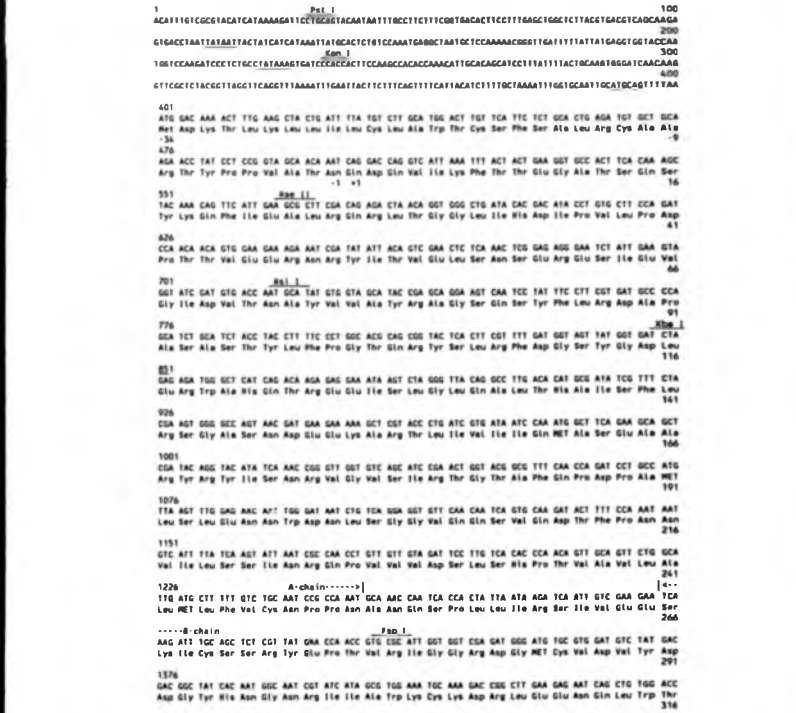
Figure 19 *Nucleotide sequence of the insert of pQK3* The coding strand sequence of pQK3 is numbered (bold type) for reference. The deduced amino acid sequence of the open reading frame is shown in bold type above the corresponding nucleotide sequence and is numbered for reference. The frameshift is shown with the amino acids deduced within this frame numbered in brackets



3.6.4 Sequence analysis of pQK9

Analysis of the sequence of the insert of pQK9 by translation into amino acid sequence shows the insert contains a single long open reading frame encoding 563 amino acids and is shown in Figure 20. The amino acid sequence determined for the abrin-related probe is identical over its length with the sequence shown here.

The similarity between ricin and abrin suggested that the structure of the polypeptide encoded within pQK9 would be similar to preproricin. Based on a comparison with abrin C A-chain and ricin D the polypeptide encodes an abrin-related preproprotein consisting of a leader of 34 amino acids, an A-chain of 251 amino acids, a linker of 14 amino acids and a B-chain of 263 amino acids


Pst I
Sac I
Bgl II
Bam HI
Bst I
Xba I
Pst I
A-chain
B-chain

```
1451
TTG AAA AGT GAC AAG ACG ATA AGG TCC AAC GGG AAG TGT TTA ACC ACA GAA GGT TAT GCT CCA GGG AAT TAT GTC
Leu Lys Ser Asp Lys Thr Ile Arg Ser Asn Gly Lys Cys Leu Thr Thr Glu Gly Tyr Ala Pro Gly Asn Tyr Val
                                                                                              341
1526
ATG ATA TAT GAT TGT ACC TCG GCA GTA GCA GAG GCC ACT TAT TGG GAA ATA TGG GAC AAT GGA ACC ATC ATC AAT
MET Ile Tyr Asp Cys Thr Ser Ala Val Ala Glu Ala Thr Tyr Trp Glu Ile Trp Asp Asn Gly Thr Ile Ile Asn
                                                                                              366
1601
CCA AAG TCT GCC TTG GTC TTG AGT GCT GAA TCT AGT AGC ATG GGA GGG ACA CTC ACC GTG CAA ACG AAC GAA TAT
Pro Lys Ser Ala Leu Val Leu Ser Ala Glu Ser Ser Ser MET Gly Gly Thr Leu Thr Val Gln Thr Asn Glu Tyr
                                                                                              391
1676
CTA ATG CGA CAG GGC TGG CGT ACA GGG AAT AAC ACA AGC CCT TTC GTA ACT TCA ATC AGT GGG TAT TCG GAT CTC
Leu MET Arg Gln Gly Trp Arg Thr Gly Asn Asn Thr Ser Pro Phe Val Thr Ser Ile Ser Gly Tyr Ser Asp Leu
                                                                                              416
1751
TGC ATG CAA GCT CAG GGA AGT AAT GTG TGG CTG GCT GAC TGT GAT AAC AAT AAG AAG GAG CAG CAA TGG GCA CTC
Cys MET Gln Ala Gln Gly Ser Asn Val Trp Leu Ala Asp Cys Asp Asn Asn Lys Lys Glu Gln Gln Trp Ala Leu
                                                                                              441
1826
TAC ACA GAT GGG TCT ATA CGT TCA GTG CAA AAT ACA AAC AAC TGT TTA ACT TCT AAA GAC CAC AAA CAA GGA TCT
Tyr Thr Asp Gly Ser Ile Arg Ser Val Gln Asn Thr Asn Asn Cys Leu Thr Ser Lys Asp His Lys Gln Gly Ser
                                                                                              466
1901
CCC ATT GTC CTG ATG GCT TGC AGC AAT GGA TGG GCT AGT CAA AGA TGG TTG TTT AAA AAT GAC GGT AGC ATT TAT
Pro Ile Val Leu MET Ala Cys Ser Asn Gly Trp Ala Ser Gln Arg Trp Leu Phe Lys Asn Asp Gly Ser Ile Tyr
                                                                                              491
1976
AAT TTA CAT GAC GAC ATG GTG ATG GAT GTG AAA CGC TCT GAT CCA AGT CTT AAA GAG ATA ATA CTT CAT CCG TAC
Asn Leu His Asp Asp MET Val MET Asp Val Lys Arg Ser Asp Pro Ser Leu Lys Glu Ile Ile Leu His Pro Tyr
                                                                                              516
Nco I
CAT GGT AAA CCT AAC CAA ATA TGG CTT ACT TTG TTT TAA TGGCTTCATGTCTGAGGAGGCAAATGCTATCTGCCTCTGTAATGTTTA
His Gly Lys Pro Asn Gln Ile Trp Leu Thr Leu Phe TER
                                                528
2138                                                                                          2237
AATAAGTGACGGAATCTAGTCGATTCCAGAAACAGTCATGAGTTCATGCTGCCTTGTAATCTTTCTAATGTGCTTAAATAATAAATTTATGTTTTAGC
```

Figure 20 *Gene sequence of the preproprotein* The coding sequence of pQK9 is numbered above for reference, with relevant restriction sites noted. Potential control sequences are underlined. The deduced amino acid sequence is shown beneath the corresponding nucleotide sequence and is numbered below. The N-terminal residue of the mature A-chain is numbered 1 and residues 5′ to residue 1 are numbered negatively. The 14 amino acid linker separating the C-terminus of the A-chain and the N-terminus of the B-chain is indicated

3.7 ANALYSIS OF THE ABRIN-RELATED PREPROPROTEIN

3.7.1 Untranslated regions

The 5' flanking region of the preproprotein gene is 86% A+T. This is commonly found in other plant genes (Halling *et al.*, 1985). Upstream to the start codon are two potential promoter elements (5'-TATAA/TA-3'). In animals and plants the consensus TATA box is found approximately 25bp upstream of the cap site and is believed to act as a recognition site for RNA polymerase II.

In animals a consensus sequence GG(C/T)CAATCT found 80-100bp upstream to the cap site is known to be important to transcription. Many plant genes however lack a CAAT box or the sequence is less well conserved. In some plant genes a different box is found with consensus CATC. In other genes, including zein, the consensus $(C/T)A_{2-5}(G/T)NGA_{2-4}(C/T)(C/T)$ described as the AGGA box is found in this region although the significance of this sequence is not yet determined. The sequence shown here does not contain a recognisable CAAT, CATC or AGGA box.

A sequence 5'-CATGCAGT-3' is found at position -5. Alternating purines and pyrimidines in the 5' flanking regions of genes expressed in cotyledon tissue are termed RY repeats and are found within 300bp of the cap site in seed lectin genes (Hoffman and Donaldson, 1985). These RY repeats have been implicated in the regulation of seed lectin genes and it is postulated that by promoting Z-DNA formation near the cap site the accessibility of RNA polymerase II to the transcription start site is improved (Kilpatrick *et al.*, 1984). Of 21 legume seed genes 20 contained at least 7bp of identity with the consensus RY repeat CATGCATG (Dickenson, 1988). In the soybean lectin gene the sequence 5'-CATGCATG-3' is found at position -5 (Goldberg *et al.*, 1989).

Downstream to the stop codon are two potential poly-A signal sequences (5'-AATAA$^{G}/_{T}$-3'). All the ricin toxin and RCA

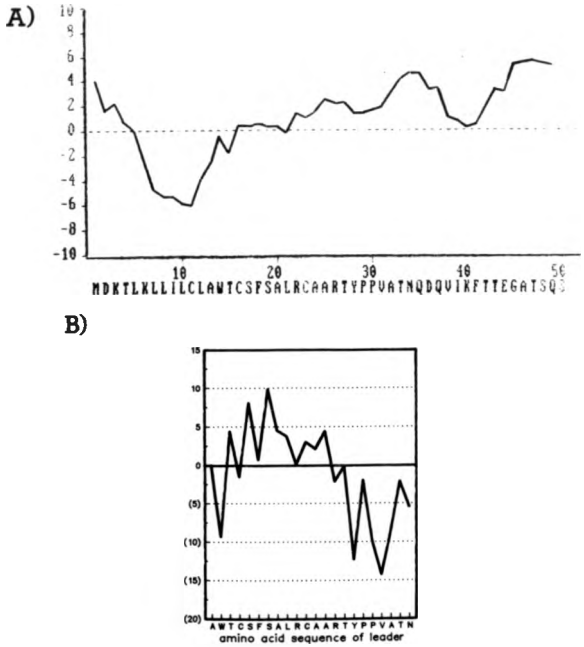
genes sequenced also contain two potential poly-A signal sequences downstream to the termination codon. In the sequence obtained from cDNA clones of ricin and RCA (Lamb *et al.*, 1985 and Roberts *et al.*, 1985) the polyadenylation signals are at 55 and 118 nucleotides downstream from the stop codon. In the preproprotein the polyadenylation signals are at 48 and 127 nucleotides downstream of the stop codon.

3.7.2 Signal sequence analysis

The leader sequence of the preproprotein consisting of 34 amino acids, was analysed using the analogue described by Parker *et al.* (1986) using a window of 7 and the result is shown in Figure 21A. A signal sequence is predicted to extend from Met-34 to Ser-16 with cleavage after the Ser-16. The N-terminus is basic, followed by a hydrophobic core extending from Leu at -31 to Thr at -20.

A method described by von Heijne (1985) was used to predict the signal sequence cleavage site and this is shown in Figure 21B. This also predicts the signal will be cleaved after the serine residue at position -16. Cleavage after serine at position -16 satisfies the current model for signal sequences (von Heijne, 1989): the N-terminus has a net positive charge and is less than 6 residues long, the hydrophobic core is more than 5 residues but less than 20 residues long, the carboxy terminus has a small non-polar residue at position -3 (serine) and at position -1 (serine) and proline is absent from residues -3 to +1.

Figure 21 *Analysis of the preproprotein leader for a signal sequence* Analysis of the leader sequence for the site of cleavage of a signal sequence was determined by the method described by (A) Parker *et al.*, 1986 which determines charged (positive value) and hydrophobic domains (negative value), and by (B) von Heijne, 1986 which gives a high positive value for potential cleavage sites



In a comparison of 136 eukaryotic signal sequences where the cleavage site has been characterised, cleavage occured after serine, cysteine or threonine in 24% (Watson, 1984) and alanine is the most common residue found in the +1 position after signal cleavage (von Heijne, 1984) which is the case here. If the site postulated for signal sequence cleavage is correct the protein must undergo further N-terminal processing to generate the mature form of the protein. This post-translational processing may by carried out by a specific protease which must recognise some feature of the cleavage domain. There is no similarity between the leader of ricin and the leader described here and there is no apparent consensus between the leaders of other ribosome-inactivating proteins which have been sequenced except for the start methionine and the Asn residue at position -1. The strong conservation of an asparagine residue at the carboxy-terminus of the leader sequences might indicate the specificity of the enzyme involved in the post-translational modification to generate the mature N-terminus of the abrin-related gene.

3.7.3 Analysis of the A-chain sequence

Overview

The A-chain of the abrin-related gene is 251 amino acids long assuming the N-terminus is glutamine as determined for abrin C and the C-terminus is asparagine from comparison with abrin C A-chain and the ricin toxins. The abrin-related A-chain contains a potential glycosylation site at Asn196, unlike abrin C A-chain which is unglycosylated. The A-chain sequence is 49% G+C and there is slight bias for A or T at the wobble position.

Arginine versus lysine bias

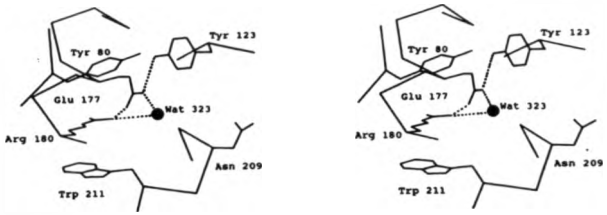
There is a bias towards arginine versus lysine with a ratio of 19:3. London and Luongo (1989) noted that many toxins exhibited an arginine versus lysine bias. Ricin A-chain has considerable bias to arginine and the A-chain sequence reported

here shows similar character with 19 arginine and only 3 lysine residues. Changes between arginine and lysine are relatively conservative. However, since arginine codons are GC rich and lysine codons are AT rich there may be some selection to maintain a high GC% composition. This is not borne out statistically however therefore maintenance of percentage GC composition alone cannot explain the bias. Arginines may be preferential for the conformation of the protein or may improve resistance to proteolysis.

The proposed active site residues

The structure of the proposed active cleft in ricin is known (Montford *et al.*, 1987). The hydroxyl of Tyr80 makes a 2.5 Å hydrogen bond to the oxygen of Gly121 whilst the other invariant residues form a supporting network structure. The side chain of Arg180 lies parallel to the plane of the indole ring of Trp211. The guanidium protrudes beyond the Trp ring where N_{H1} forms a strong hydrogen bond to the oxygen of Asn78. N_{H2} appears to donate two bonds, one to an active site water molecule and a second to O_{E2} of Glu177. The other oxygen of Glu177 forms two hydrogen bonds, one to water and the other to the hydroxyl of Tyr123 (Robertus, 1991). The residues Asn78, Arg134, Gln173, Glu208 and Asn209 in ricin are conserved active site amino acids and may play less specific roles in the catalytic mechanism or in maintaining the active site structure. Glu208 hydrogen bonds to Gln173, forms an ion pair with Arg134, and contacts the side chain of Glu177. Glu173 bonds to a trapped water molecule and anchors Glu208. Asn78 and Asn 209 sit on the molecular surface at the edge of the cleft (Robertus, 1991). Figure 22 illustrates the proposed active cleft of ricin.

Figure 22 *The active site cleft of ricin A-chain* The model includes invariant and conserved groups in the active site cleft (taken from Robertus, 1991)



Several of the amino acids with side chains in the cleft, are invariant in the ribosome-inactivating proteins. In ricin these invariant residues are Tyr80, Tyr123, Glu177, Arg180 and Trp211 and these same residues are conserved within the sequence of the A-chain reported here. Of the other invariant residues found in the ribosome-inactivating proteins, which are Tyr21, Arg29, Leu144 and, Ala178 in ricin, all are conserved in the A-chain sequence reported here. The conserved active site residues, equivalent to Asn78, Arg134, Glu208 and Asn209 in ricin, are also found in this A-chain sequence.

In addition, amino acids are conserved at certain positions in *E. coli* RNase H, Rous sarcoma virus reverse transcriptase and ricin D A-chain which include the residues equivalent to Ala165, Leu214 and Pro229 in ricin. RNA binding is the common function shared by these proteins and these amino acids may be important for the enzymatic function of RNA-binding proteins (Ready *et al.*, 1988). Again these residues are conserved in the A-chain sequence reported here although they are not invariant within all ribosome-inactivating proteins.

Site directed mutagenesis has been used by several workers to determine the effect on catalytic activity. Generally mutations in ricin A-chain have had relatively limited effects on biological activity compared to key residue mutations in other enzymes (Robertus, 1991). In ricin, a mutation of Trp211 to Phe led to a nine fold drop in activity (Bradley and Mcquire, 1990). Mutation of the Arg180 to His reduced activity by 1000 fold (Frankel *et al.*, 1990), alteration to Gln reduced activity by 200 fold (Robertus, 1991) whereas alteration to Lys has minimal effect (Frankel *et al.*, 1990). These results indicate that a positive charge is required at position 180 and not a guanidinium group *per se*.

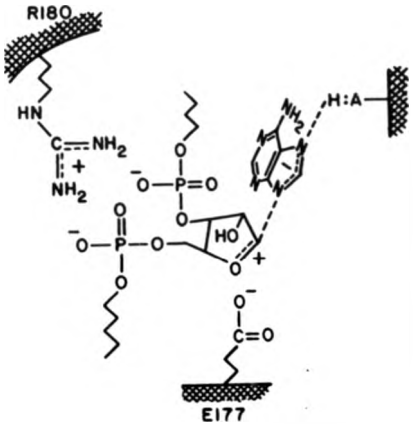
Glu177 has been mutated to Ala with only a 20 fold loss in activity and to Asp with a 80 fold drop in activity. Hovde *et al.* (1988) found that in *E. coli* Shiga-like toxin I, a mutation of Glu167, equivalent to Glu177 in ricin, to Asp reduced activity by 1000 fold. A double mutation in ricin A-chain

where Glu177 is altered to Ala, and Glu208 is altered to Asp, reduced activity by 1000 fold. Mutation of Glu208 alone does not alter activity significantly therefore it has been suggested that Glu208 can substitute for Glu177 when the carboxylate of Glu177 is removed (Robertus,1991)

Frankel *et al.* (1989) selected for mutations in ricin A-chain by loss of lethality when expressed in yeast. Eight of the nine mutants they isolated had single amino acid changes in the proposed active site. These mutations were Glu177 to Asp, Glu177 to Lys, Trp211 to Arg, Gly212 to Glu, Gly212 to Trp, Ser215 to Pro and Ile252 to Arg.

The current model for the *N*-glycosidation reaction assumes protonation of the leaving adenine group with stabilisation of the oxycarbonium ion which would develop during the transitional state of the ribose. The hypothesis is supported from model experiments using kinetic isotope effects to investigate hydrolysis of the adenine base of AMP by a nucleoside (Mentch *et al.*, 1987 and Giranda *et al.*, 1988). During the transition state the leaving adenine is protonated, the ribose ring has carbonium character, there is partial bonding between the base and the ribose and nucleophilic attack at the C1 of ribose by a participating water molecule. Based on this evidence and the results of mutagenisis of ricin A-chain a mechanism for the enzymatic reaction of ricin A-chain has been made (Robertus, 1991) and is summarised in Figure 23.

Figure 23 *A schematic diagram of the proposed ricin A-chain mechanism* The adenine ring is shown of the substrate is shown in the syn conformation, with partial breakage. Oxycarbonium ion development is shown on the ribose, and this stabilises by ion pairing with Glu177. Arg180 is shown binding the phosphate backbone of the RNA. The attacking water molecule is positioned close to the scissile glycoside linkage. A:H represents an unspecified residue which hydrogen bonds to the leaving adenine



The ribosome is recognised and bound by several contacts of which none are of major significance alone. The susceptible adenine ring is distorted into the high energy *syn* configuration by the many favorable interactions. The juxtaposition of Glu177 to the developing oxycarbonium ion on the ribose enhances bond breakage. Protonation of the leaving adenine is from several donors such as Tyr80 and Tyr123. Arg180 forms an ion pair with a substrate phosphate, thereby stabilising substrate binding and contributing to the energy needed to distort the susceptible adenine conformation.

Recently Evensen *et al.* (1991) used PCR to isolate two complete abrin-related A-chain sequences. These two sequences had 84% identity with each other and comparison with the A-chain sequence reported here shows a similar degree of similarity.

Cysteine residues

In ricin the A-chain and B-chain are linked by a single disulphide bond between residue 259 of the A-chain and residue 4 of the B-chain. In the abrin-related A-chain there is a single cysteine residue which corresponds to Cys 259 in ricin A-chain.

Summary

The A-chain sequence of the abrin-related preproprotein has all the features characteristic of ribosome-inactivating proteins. At positions which have been shown to be important for calalytic activity of ricin A-chain there is 100% identity between the A-chain sequence reported here and ricin A-chain.

Recently a molecular model for abrin C A-chain has been described (Collins *et al.*, 1990) which was generated by fitting the primary sequence of abrin C A-chain to the backbone structure for ricin A-chain. The overall structure of the abrin model was very similar to that of ricin despite the 17 additional residues in ricin A-chain. The deletions are easily accommodated in the abrin molecule. The two major deletions,

centred on ricin residues 105 and 151, lie on the surface of the molecule, the five single-deletions and the single insertion do not significantly perturb the peptide and the internal packing of the molecule is not disrupted. The close similarity between the primary structure of abrin C A-chain with the A-chain reported here, and the similarity between the structure of abrin C A-chain and ricin A-chain suggests that analogies drawn between ricin and the abrin-related preproprotein will be valid.

3.7.4 Analysis of the linker

The linker in the cloned abrin-related gene is 14 amino acids long. The linker in type II ribosome-inactivating proteins is believed to maintain the protein in an inactive form until it is sequestered within the protein storage bodies (Lord, 1985). The linker is then cleaved to release the subunits of the heterodimer. Proricin has no *N*-glycosidase activity but is still functional as a lectin (Richardson *et al.*, 1989). The linker region must significantly disrupt the active site of the A-chain, or prevent access by the substrate to the cleft within the A-chain, but not interfere with the galactose binding sites of the B-chain.

Unexpectedly the linker in the cloned abrin-related gene has a potential glycosylation site. The ricin toxins and RCA do not have a glycosylated linker. The glycosylation of the linker may stabilise the polypeptide in some way during transport to the protein storage bodies or the increased bulk of the linker domain may contribute to the folding of the polypeptide. A glycosylated linker is found in concanavalin A (Con A), a lectin which constitutes up to 15% of the protein in the cotyledons of *Canavalia ensiformis* (Carrington *et al.*, 1985). Con A also undergoes post-translational cleavage of a glycosylated linker.

The linker in the gene described here is also unusual in that it does not end with an Asn residue. In the ricin toxins,

in RCA and in Con A all the points which must be cleaved to produce the mature protein have an Asn residue to the N-terminal side of the point of cleavage indicating the specificity of the enzyme involved.

3.7.5 Analysis of the B-chain

Overview

The B-chain of the preproprotein is 263 amino acids long. The serine was selected as the N-terminus based on comparison with ricin and RCA to conserve the positioning of cysteine residues and serine versus alanine (in ricin and RCA) is a relatively conservative change. Sequencing of the N-terminus of the B-chain of abrin toxins has been unsuccessful to date (personal communication, Dr E.J. Wawrzynczak, Institute of Cancer research, Surrey, UK). This has been due to blocking either in nature or as a consequence of the sequencing procedure. The glutamate residue is more likely to be blocked in nature but without protein sequence information the first residue of the B-chain cannot be confirmed.

The sequence for the B-chain reported here is only the second to be reported apart from those from *R. communis*. The structure of the B-chain of ricin has been determined (Montford *et al.*, 1987) and from this, the residues critical to correct folding and activity have been indicated.

Cysteine residues

Ricin B-chain contains 4 intrachain disulphide bonds. The cysteine residues in the abrin-related B-chain sequence are conserved with ricin and are at positions 4, 20, 39, 63, 80, 151, 164, 190 and 207. The cysteine residue at position 4 in the B-chain is likely to form the interchain bond with the A-chain. The N-terminus of ricin B-chain has been shown to be flexible and mobile from NMR spectra studies (Bushuev and Tonevitsky, 1989) and the X-ray crystallographic studies of crystals of ricin (Montford *et al.*, 1987) shows the N-terminus

of the B-chain lies outside the globular part of the protein. It has been suggested that the high mobility of the N-terminus of the B-chain may promote both the formation and destruction of the interchain disulphide bond. The remaining 8 cysteine residues, being in the identical positions to ricin can potentially form 4 intrachain disulphide bonds with the pairings of 20/39, 63/80, 151/164 and 190/207.

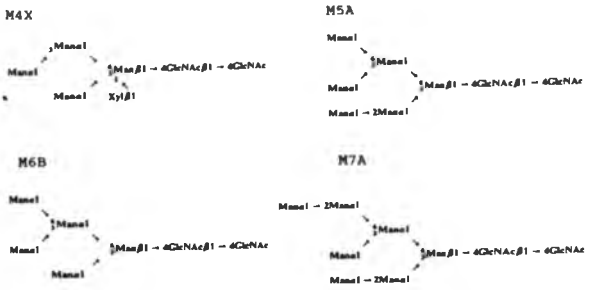
Glycosylation sites

The B-chain sequence reported here contains 2 potential glycosylation sites. The ricin toxins also contain 2 glycosylation sites in the B-chain which are at the same position as in the B-chain of the abrin-related preproprotein. The glycosylation of the B-chain has been shown to be important in the stability of the B-chain. When ricin B-chain is expressed in *E. coli*, and is therefore unglycosylated, it is unstable and aggregates rapidly (Hussain *et al.*, 1989) although initially it is soluble and active. Glycosylation has been shown to be significant for expression of B-chain in oocytes. When pre-ricin B-chain is expressed in oocytes the product is soluble, stable and functionally active (Richardson *et al.*, 1988a). However, if tunicamycin is used to inhibit glycosylation (Richardson *et al.*, 1989) or the glycosylation sites are mutated (Wales *et al.*, 1991) the product is unstable, rapidly aggregates and is devoid of lectin activity. The glycosylation of the B-chains of the type II ribosome-inactivating proteins is therefore of significance to the stability and activity of the isolated B-chain. The importance of *N*-linked oligosaccharides in maintaining native and recombinant glycoproteins in active conformations has been demonstrated for several different protein types (Fischer *et al.*, 1990 and Kretz *et al.*, 1990)

The structure of the sugar chains of the B-chain of abrin C has been determined (Kimura *et al.*, 1988b). Kimura postulated that the glycosylation sites within the abrin B-chain correspond to Asn97 and Asn136 of ricin B-chain based on the

sequence of the glycopeptides generated from pronase digestion of the B-chain. The estimated structures of sugar chains obtained from abrin C B-chain are shown in Figure 24. The two oligomannose-type sugars M7A and M6B were linked to the site corresponding to Asn 97 in ricin B-chain, and the two oligomannose-type sugars M6B and M5A and the xylomannose-type sugar M4X were linked to the site corresponding to Asn 136 in ricin B-chain.

Figure 24 *The structure of the sugar chains of abrin C B-chain*
The structure of the oligosaccharide side chains determined by Kimura *et al.*, 1988, are shown below. Abbreviations are Man, mannose; Xyl, D-xylose; GlcNAc, *N*-acetylglucosamine



Evolution of the B-chain

Ricin B-chain is postulated to be a product of gene duplication. Structural homology within the ricin B-chain show that the two halves exhibit a relationship in the pattern of disulphide bridges, loop size and sequence homologies (Villafranca and Robertus, 1980). Rutenber *et al.* (1987) have analysed structural homology within the B-chain to a more extensive degree and postulate that the B-chain of ricin is a product of a series of gene duplications of an ancient galactose-binding peptide of approximately 40 residues. Analysis of primary and tertiary structure has revealed that the the two halves of ricin B-chain can be divided into four peptides called the λ, α, β and γ subdomains. The lambda domains are homologous to each other but not to the other domains. The α, β and γ domains are all homologous with each other - despite considerable divergence the similarities are statistically significant.

Amino acid sequence analysis of the subdomain units of the abrin-related B-chain (Figure 25A) show that the subdomains α, β and γ are homologous and show similarity to the subdomains of ricin B-chain. As with ricin B-chain there are invariant Trp residues and strongly conserved Ile residues. Dr M.P. Ready (University of Texas at Austin, Texas, USA) determined the similarity matrix between ricin B-chain and the cloned abrin B-chain using the method of Robertus and Ready (1984). Briefly, the aligned sequences are scrambled several hundred times; an average number of identities and standard deviations are determined for random sequences of the same composition as the test sequences; a variable gap penalty is then applied which gives large penalties for gaps which produce few new identities and small penalties for gaps which produce many; a similarity index is then determined for the aligned sequences, based on the gap-corrected number of identities between them, in standard deviations above the mean for randomised sequences. As shown in Figure 25B the similarity between the subdomains of ricin B-chain and the cloned abrin B-chain is strong.

Figure 25 *Alignment of the subdomains of ricin B-chain and the abrin-related B-chain based on amino acid sequence similarity* (A) Amino acid sequence alignment of the subdomains of ricin B-chain (RTB) with the subdomains of the B-chain of the preproabrin (ATBKW). The subdomains correspond to residues as follows: 1α residues 17-60 (RTB), 18-61 (ATBKW); 1β, 61-101,62-102 (ATBKW); 1γ, 104-136 (RTB), 105-137 (ATBKW); 1λ, 1-16 (RTB), 1-15 (ATBKW); 2α, 151-183 (RTB), 152-184 (ATBKW); 2β, 187-226 (RTB), 188-227 (ATBKW); 2γ, 228-262 (RTB), 229-263 (ATBKW); 2λ, 136-150 (RTB), 137-151 (ATBKW). (B) Similarity indices for all possible comparisons, calculated as described (Robertus and Ready, 1984), measured in standard deviations above the mean for random matches and including gap penalties

A)

Alignment Based on Amino Acid Sequence Similarity

```
ATBKW   1α    DGMCVDVYDDGYHNGNRIIAW--KCKDRLEENQLWTLKSDKTIRS
        2α    SDLCMQA---------QGSNVWLADC-DNNKKEQQWALYTDGSIRS
RTB     1α    NGLCVDVRDGRFHNGNAIQLW--PCKSNTDANQLWTLKRDNTIRS
        2α    YGLCLQA---------NSGQVWIEDC-SSEKAEQQWALYADGSIRP

ATBKW   1β    NGKCLTT---EGVAPGNYV-MIYDCTSAVAEATYWEIWDNGTIIN
        2β    TNNCLTS---KDMKQGSFI-VLMAC-SNGWASQRWLFKNDGSIYN
RTB     1β    NGKCLTT---YGYSPGVYV-MIYDCNTAATDATRWQIWDNGTIIN
        2β    RDNCLTS---DSNIRETVV-KILSC-GPASSGQRWMFKNDGTILN

ATBKW   1γ    SALVLSAESSSMGGTLTVQTNEYLM-------RQGW-RTGN
        2γ    DDMVMDVKR--SDPSLK-EIILHPY--HGKPNQIW-LTLF
RTB     1γ    SSLVLAATSGNSGTTLTVQTNIYAV-------SQGW-LPTN
        2γ    SGLVLDVRA--SDASLK-QIILYPL--HGDPNQIW-LPLF

ATBKW   1λ    SKICSSRYEPTVRIGGR
        2λ    NTSPFVTSISGY
RTB     1λ    ADVCM-DPEPIVRIVGR
        2λ    NTQPFVTTIVGL
```

B)

RTB vs. ATB	Alpha	Beta	Gamma
Subdomain 1	15.49	16.86	10.30
Subdomain 2	13.12	8.00	14.81

Galactose binding sites

Ricin B-chain has been shown to bind two galactose molecules in a non-cooperative manner (Hooston and Dooley, 1982) and the molecular interactions at the binding sites has been determined from X-ray crystallographic data (Montford *et al.*, 1987). The galactose binding sites are found in non-homologous domains within ricin B-chain, namely the 1α and the 2δ domains. Montford and co-workers postulate that the ancestor of the modern B-chain was a peptide resembling the 1α domain. The primordial molecule probably existed as a trimer linked by a Trp-Ile interaction. Gene duplication and fusion then formed the αβδ-molecule which allowed specialisation of the β subunit for a structural role, and the αβδ molecule could bind sugars at both the α and δ domains. The molecule was stabilised by the addition of the λ peptide and a further gene duplication generated the current $(\lambda\alpha\beta\delta)_2$ motif. This duplication blocked access of galactose to the 1δ domain and although there is still solvent access to the 2α binding domain this has lost binding ability. It is suggested that selection for the two-domain structure involved the spacing of the binding sites for cell surface binding (Rutenber *et al.*, 1987).

From the similarity indices determined for the abrin B-chain and ricin B-chain (Figure 25B) the 2β domain is the least constrained. Reduced similarity is consistent with the location of 2β in the protein, where it is exposed to the solvent and appears to play no crucial role other than contribution to overall folding.

The structure of the two functional binding domains in ricin B-chain is similar. The sugar molecule lies in a pocket formed on one side by an aromatic residue (Trp37 in 1α and Tyr248 in 2δ) and the other by a kink consisting of Asp-Val-Arg. The aromatic side chains provide a flat binding surface for galactose but the interaction does not involve specific binding. The galactose is hydrogen bonded by the homologous amides Asn46 and Asn255, which are in turn stabilised by hydrogen bonding to Asp22 and Asp234 (Montford *et al.*, 1987).

The sequence Gln-X-Trp is common to five of the ricin B-chain subdomains and the Trp is invariant. The Trp residues stabilise the carboxy-terminal loop of each subdomain and stabilises the subdomain contacts within each domain by Trp-Ile van der Waals' contacts with the highly conserved downstream Ile residue (Robertus, 1991).

Recently, Rutenber and Robertus (1991) published a refined model for the structure of ricin B-chain at 2.5Å. This structure differed from that previously published (Montford *et al.*, 1987). An alignment between the cloned abrin B-chain sequence and ricin B-chain, based on the structural position of residues from the refined model of ricin B-chain, is shown in Figure 26A. The abrin B-chain molecule retains the vital folding core residues and the residues involved in the hydrophobic core are conserved apart from a Leu to Ala change in the 1a domain. The residues which make specific contact with the sugars are also retained except for the His to Tyr change in the 2δ domain and Glu for Ile in the 1α domain. The presence of Ile in the 1 domain is unlikely to be important since the functional domain of ricin B-chain has an Ile at this position.

The similarity indices of the alignment shown in Figure 26A were determined by Dr M.P. Ready (University of Texas at Austin, Texas, USA) and are shown in Figure 26B. The similarity indices for the subdomains of the abrin B-chain are consistently less similar amongst themselves than the subdomains of ricin B-chain. Determining the subdomain structure of the abrin B-chain would have been difficult without comparison with ricin B-chain

Figure 26 *Alignment of the subdomains of ricin B-chain and the abrin-related B-chain based on the structural position in ricin B-chain* (A) Amino acid sequence alignment of the subdomains of ricin B-chain (RTB) with the B-chain of the preproabrin (ATBKW) based on the structural position in RTB (Rutenber and Robertus, 1991). (I) indicates cyteines at the base of the loop, (↓) indicates residues critical for the hydrophobic core, (⌂) indicates residues involved in the hydrophobic core and (*) indicates the residues of 1α and 2β involved in sugar binding. The residues in each subdomain are listed in the legend to Figure 25. (B) Similarity indices (s.i.) of ATBKW and of (C) RTB for all possible comparisons, calculated as described (Robertus and Ready, 1984) measured for standard deviations above the mean (σ) for random matches and including gap penalties

A)

```
                    ↓▲*          ⌂*⌂* ↓       **  ▲          ▲
ATBKW   1α   DGMCVDVYDDGYHNGNR-IIAWKCKDRLEENQLWTLKSDKTIRS
        2α   SDLCMQA-Q---G--SN-VWLADCDNNKK-EQQWALYTDGSIRS
RTB     1α   NGLCVDVRDGRFHNGNA-IQLWPCKSNTDANQLWTLKRDNTIRS
        2α   YGLCLQA-N---S--GQ-VWIEDCSSEKA-EQQWALYADGSIRP

ATBKW   1β   NGKCLTTE-G-YAPGNY-VMIYDCTSAVAEATYWEIWDNGTIIN
        2β   TNNCLTS-KDH-KQ-GSPIVLMACSNG-WASQRWLFKNDGSIYN
RTB     1β   NGKCLTTY-G-YSPGVY-VMIYDCNTAATDATRWQIWDNGTIIN
        2β   RDNCLTS-DSN-IR-ETVVKILSCGPA-SSGQRWMFKNDGTILN

ATBKW   1γ   SALVLSAE-SSSM-GGT-LTVQTNEYL-M-RQGWRTGN
        2γ   DDMVMDVKRSDPS-LKE-IILHPYH-G-KPNQIWLTLF
RTB     1γ   SSLVLAAT-SGNS-GTT-LTVQTNIYA-V-SQGWLPTN
        2γ   SGLVLDVRASDAS-LKQ-IILYPLH-G-DPNQIWLPLF
```

B)

S.I.(σ)	1 α				
1 β	2.77	1 β			
1 γ	0.07	0.31	1 γ		
2 α	5.02	1.78	2.63	2 α	
2 β	3.02	4.89	0.01	5.79	2 β
2 γ	3.68	(1.54)	2.01	2.77	0.70

C)

S.I.(σ)	1 α				
1 β	2.45	1 β			
1 γ	1.96	1.22	1 γ		
2 α	10.19	4.75	3.36	2 α	
2 β	3.16	7.85	2.38	5.17	2 β
2 γ	5.72	0.05	6.65	1.90	0.10

The motif for a functional binding site is evident at the 1α domain of the cloned abrin B-chain. In ricin this domain is termed the low affinity site because it can bind to galactopyranosides but not *N*-acetylgalactosamines (Hatakeyama *et al.*, 1989). From the sequence comparison the B-chain of the abrin clone was expected to have a low affinity binding site.

In the 2δ subdomain of the abrin B-chain there is only one significant difference in the residues considered important to galactose binding and this is a histidine as opposed to tyrosine in ricin B-chain. In ricin D the 2δ domain is a high affinity site, binding to both galactopyranosides and *N*-acetylgalactosamines (Hatakeyama *et al.*, 1989). A histidine residue is found at the equivalent position in ricin E and in RCA. The significance of the histidine is not determined. Histidine also has an aromatic side chain but it is unlikely that histidine can substitute for tyrosine or tryptophan as a 'sugar platform' (personal communication, Dr J.D. Robertus, University of Texas at Austin, Texas, USA).

RCA can bind to only one molecule of lactose per B-chain (Podder *et al.*, 1974). RCA has a similar motif to ricin D in the 1α subdomain therefore it may be a consequence of the histidine in the 2δ domain that has led to loss of binding at this site. The interaction within the tetramer of RCA is unknown, therefore in the native RCA, solvent access to the second binding site on each B-chain may be prevented or the interactions involved in the quaternary arrangement of the protein disrupts one of the sites. The B-chains of APA have been isolated and shown to have very low cytoagglutinating activity of approximately 0.9% compared to intact APA (Islam and Funatsu, 1988) suggesting monovalency.

Based on the alignments described above the cloned abrin B-chain is expected to have a low affinity site in the 1α domain. The site in the 2δ domain is expected to be equivalent to the binding site in the 2δ domain of ricin E B-chain.

3.7.6 Comparison with ricin and RCA

Overview

An alignment between the preproprotein sequence, the ricin toxins and RCA is shown in Figure 27. The A-chain of the preproprotein exhibits 83% identity with the A-chain of abrin C, 42% identity with the ricin toxin A-chains and 36% identity with RCA A-chain. The abrin-related A-chain is one residue longer than abrin C A-chain due to an additional glycine residue at position 204.

The B-chain of the preproprotein exhibits 60.8% identity with ricin D B-chain and 60.2% identity with RCA B-chain. The preproprotein leader shows no significant similarity to the leaders of the ricin toxins or RCA apart from the Asn residue at -1. There is some similarity to the linkers of the ricin toxins and RCA.

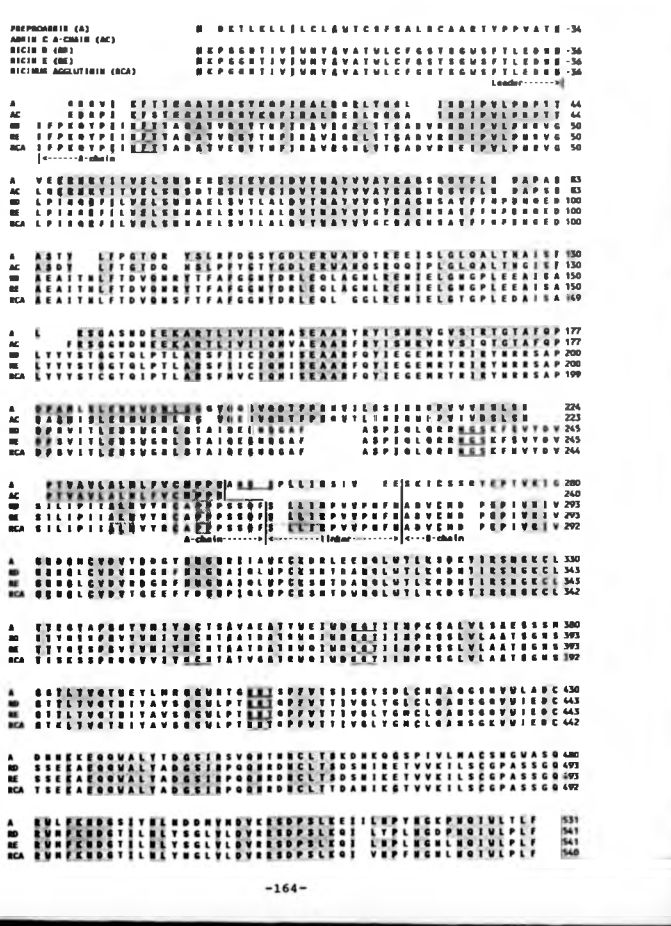
Toxins versus agglutinins

Ricin toxins are closely related to RCA and therefore an abrin toxin would be expected to be closely related to an APA. Despite the close similarity between these two types of protein some residues must be responsible for determining the enzymatic and structural differences exhibited by the toxins versus agglutinins. Previous comparisons have shown that ricin D differs from RCA at only 57 positions (Roberts *et al.*, 1985). At the positions where these differences occur a comparison was made between the sequence described here and with the A-chain of abrin C, the ricin toxins and RCA.

Within the A-chain sequences there are 16 positions where the ricin toxins and RCA differ. Of these 16 positions, residues are conserved between the preproprotein and abrin C alone at 4 positions. At 9 positions the residue is conserved between the preproprotein and all the toxins but at only one position is the residue conserved between the preproprotein, abrin C and RCA. At 2 positions the residues are not conserved between the five sequences.

Figure 27 *Alignment of the preproabrin with abrin C, ricin toxins and RCA* An alignment between the preproabrin (A), abrin C (AC), ricin D (RD), ricin E (RE) and RCA is shown using the single amino acid code; each sequence is numbered for reference according to the published sequences. The leader, A-chain, linker and B-chain sequences are marked. Residues conserved with the preproabrin are indicated by shaded boxes. *N*-glycosylation sites are underlined

-facing 164-


PREPROABRIN (A)
ABRIN C A-CHAIN (AC)
RICIN D (RD)
RICIN E (RE)
RICINUS AGGLUTININ (RCA)
Leader
A-chain
B-chain

Within the B-chain sequences, differences occur at 41 positions. At these positions the preproprotein matches a ricin toxin at 20 positions but at no position does the preproprotein match only RCA. At 20 positions the residues are not conserved between all four sequences.

N-terminal sequencing of an APA B-chain of Korean origin (Wood *et al.*, 1991) generated the sequence:

V-V-X-G-S-I-I-S-S-

This is similar to the N-terminal sequence determined for an APA B-chain of Bangladeshi origin (Islam and Funatsu, 1988).

Protein sequencing of a carboxy-terminal CNBr fragment from an abrin toxin A-chain of Korean origin (personal communication, Dr E. Wawrzynczak, Institute of Cancer Research, Surrey, UK) generated the sequence:

*

D-V-X-G-S-D-P-S-L-X-Q-I-I-L-H-P-Y-H-G-K-P-N-Q-I-

This is similar to the carboxy terminus of the B-chain sequence of the preproprotein reported here and has a His residue at the position postulated to be involved in potential galactose binding at the 2g domain of the cloned abrin sequence (*).

Based on the closer similarity of the preproprotein to the ricin toxins than to RCA and the dissimilarity between the N-terminal sequence data available on APA, the preproprotein is abrin-like as opposed to APA-like.

Interchain interactions

The A-chain and B-chain of ricin are linked by a single disulphide bond. Youle *et al.* (1983) have shown that this disulphide bond is not required for cytotoxicity except at low concentrations when the disulphide bond maintains the subunits in close juxtaposition. The association between the subunits of ricin has been shown to be via hydrophobic interactions. The

dissociation constant was determined as 1.72×10^{-6}, and the entropy and enthalpy values were positive.

In ricin the interchain interactions between the A-chain and B-chain are shown in Table 4. The residues in the A-chain which are conserved with the sequence reported here are equivalent to Tyr183, Leu207, and Pro250. Within the B-chain the residues which are conserved are eqivalent to Phe262 and Phe140. The high percentage identity between abrin C A-chain and the similarity in the tertiary structure of abrin C A-chain to ricin A-chain therefore suggests a similar hydrophobic interaction between the A-chain and B-chain of the protein desribed here. The 3-dimensional structure of ricin has shown that in addition to the driving hydrophobic interactions there are also some polar interactions which occur over a small contact area. In the A-chain of the abrin-related gene there is conservation of polar residues in the domains involved in the contact area. The tertiary structure of abrin C A-chain is similar to ricin in these domains therefore it seems likely that these polar interactions extend over a relatively small area in the abrin-related protein. It is postulated that the interaction between the subunits relies on both hydrophobic and polar interactions so that the two chains can be separated. The subunits could not be separated if the interaction consisted of entirely hydrophobic bonding (Robertus, 1991).

3.7.7 Summary of sequence analysis

Sequence analysis has shown that the preproprotein is closely related to the ricin toxins. Based on the closer similarity to the toxin sequences than to the RCA sequence, at the few residues where differences are seen, the preproprotein most closely resembles a toxin and not an APA. The protein will be termed as an abrin in future discussion.

Table 4 *Interchain interactions in ricin*

Hydrophobic residues		Polar interactions	
A-chain	B-chain	A-chain	B-chain
Tyr183	Phe262	Arg258	Ala1
	Pro260	Ala260	Asp2
		His40	Asp94
Leu207	Phe262	Arg234	Val141
		Glu41	Lys219
Phe240	Phe140	Gln182	Asn220
	Phe262	Ile249	Asn220
		Ile252	Asn220
Ile247	Phe140	Arg235	Phe262
Pro250	Phe218		
	Pro260		
Ile251	Pro260		
	Phe262		

3.0 A-CHAIN EXPRESSION IN *E. COLI*

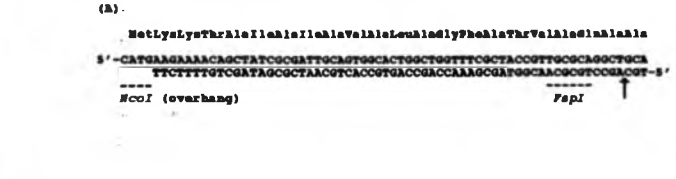
3.0.1 Periplasmic expression

Periplasmic expression construct

The signal sequence used to direct the nascent A-chain polypeptide to the periplasmic space of *E. coli* was the OmpA signal peptide. Omp A is the major outer membrane protein of *E. coli*, present at a concentration of 10^5 per cell (Beck and Bremer, 1980). The signal peptide of Omp A has been used to export a wide variety of eukaryotic proteins to the periplasmic space (Takagi *et al.*, 1988). These secretion cloning vectors can have benefits over a cytoplasmic expression system: the mature secreted protein can have the correct native amino-terminal amino acid residue, protease activity in the periplasm is considerably less than in the cytoplasm and disulphide bond formation has been shown to occur simultaneously with secretion (Marston, 1986).

A double stranded oligonucleotide encoding the OmpA signal was synthesised and is illustrated in Figure 28B. An additional codon encoding alanine was included at the 3' end: the first residue of the mature A-chain sequence is glutamine which is rarely found at the +1 position after prokaryotic signal sequences (Watson, 1984). The inclusion of the alanine residue should improve the efficiency of cleavage of the signal *in vivo*. The oligonucleotides were designed such that, when annealed, an *NcoI* overhang was generated at the 5' end and the 3' end was blunt.

Figure 28 *Oligonucleotide primer design for the OmpA signal sequence and for recover of the A-chain sequence by PCR* (A) The primary sequence of the Omp A signal sequence is shown with the nucleotide sequence of the synthesised primers below. Restriction sites are marked and the cleavage site is indicated (↑). (B) The sequence of the oligonucleotide primers used in PCR on pQK9 DNA template to recover the mature A-chain sequence. The sequence of the A-chain from which the primers were designed is numbered according to Figure 27. The orientation of the primers is indicated with the primer 9-AR representing complementary DNA. The linker regions, to generate restriction endonuclease recognition sites are underlined



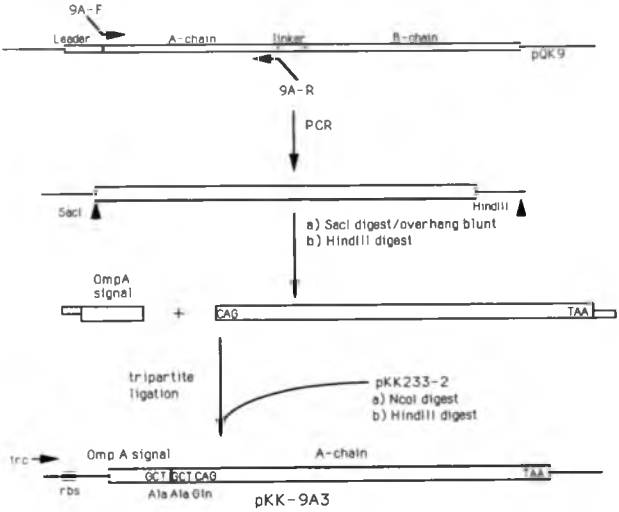
(B) **Primer 9-AF**

```
                    1                                   8
                    Gln Asp Gln Val Ile Lys Phe Thr
5'- CACGGATCCCTCGAGCT CAG GAC CAG GTC ATT AAA TTT ACT AC -3'
      BamHI     SacI
```

Primer 9-AR

```
    246                 251
    Val Cys Asn Pro Pro Asn TER TER
3'- CAG ACG TTA GGC GGT TTA ATT ATT CGAAGGACGTCCAC -5'
                                HindIII  PstI
```

Figure 29 *Summary of the cloning strategy for periplasmic A-chain expression construct pKK-9A3*



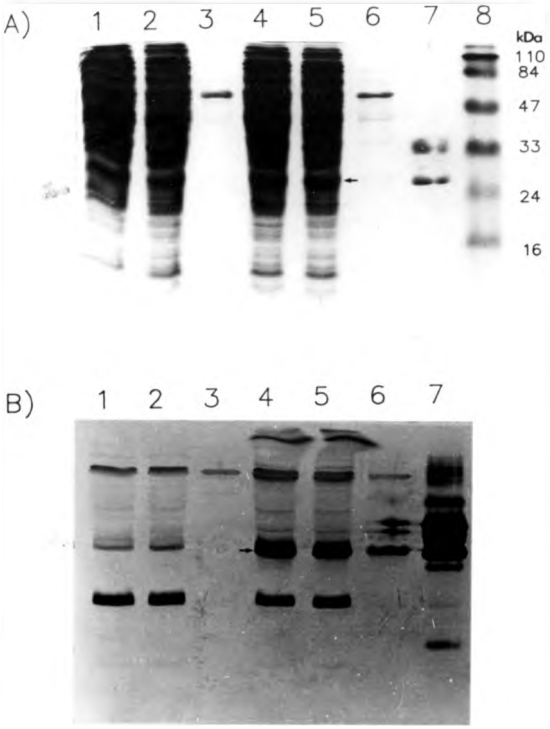
The mature A-chain sequence was recovered by PCR using linker-extended primers 9A-F and 9A-R, on pQK9 DNA template. The design of the primers is shown in Figure 28B. The PCR product therefore consisted of the A-chain sequence flanked by a 5' *SacI* site, and a 3' *HindIII* site. The final construct termed pKK-9A3 was generated by a tripartite ligation of the double stranded oligonucleotide, the A-chain which had been sequentially blunted at the *SacI* site then digested with *HindIII*, and the vector pKK233-2 which had been digested with *NcoI* and *HindIII*. The cloning strategy is summarised in Figure 29.

Periplasmic expression in *E. coli*

The results from periplasmic expression in *E. coli* are shown in Figure 30. Analysis by SDS-PAGE and Coomassie blue staining (Figure 30A) reveal a very faint band is present in the periplasmic fraction (lane 6) which migrates the same distance as native abrin C A-chain (lane 7). Careful examination of the total cell fraction (lane 4) and shocked cell fractions (lane 5) suggest an increase in the intensity of a band of approximately 28kDa compared to the non-recombinant vector control.

Western blot analysis (Figure 30B) clearly shows the presence of an immunoreactive band of 28kDa present in total (lane 4), shocked cell (lane 5) and periplasmic fractions (lane 6). There are also however two immunoreactive bands present in the non-recombinant vector cell fractions (lanes 1 and 2) indicating cross-reactivity between the anti-sera and an *E. coli* extract. This was despite preincubation of the anti-sera with *E. coli* extract. As one of these cross-immunoreactive proteins was of a similar size to the recombinant A-chain some affinity purified anti-abrin A-chain was obtained (kindly provided by Dr E. Wawrzynczak) for the majority of future Western blot analysis.

Figure 30 *Periplasmic expression of recombinant abrin A-chain* JA221 transformed with pKK-9A3 was cultured to OD_{600} of 0.8, induced with 0.1mM IPTG and then cultured for a further 3 hours. The cells were harvested and the protein fractions were prepared as described (section 2.16.2). The total, shocked cell and periplasmic fractions were analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (section 2.18.2). Lanes 1-3, total, shocked cell fraction and periplasmic fraction respectively from non-recombinant vector control; lanes 4-6, total, shocked cell fraction and periplasmic fraction respectively from JA221/pKK-9A3; lane 7, native abrin C; lane 8, prestained low molecular weight markers


A)
1 2 3 4 5 6 7 8
kDa
110
84
47
33
24
16
B)
1 2 3 4 5 6 7

It is clear from the Western blot that recombinant A-chain is being translated and some is being exported to the periplasm. The size estimate of 28kDa for this exported A-chain suggests that the signal peptide has been cleaved. If the signal peptide had not been cleaved a protein of approximately 30kDa would be expected. Lehnhardt *et al.* (1987) and Emr and Bassford (1982) have shown that the unprocessed fraction of pro-OmpA-ß-lactamase is clearly distinguishable from the processed form by 12% and 15% SDS-PAGE. There is no evidence of a doublet in either the total or shocked cell fractions which suggests that the majority of the expressed A-chain has lost the signal peptide and the A-chain seen in the shocked cell fraction is due to inefficient periplasmic shock.

Summary of periplasmic expression

Periplasmic expression was initially chosen for expression because purification from the periplasmic fraction was expected to be relatively simple due to the low number of contaminants.

The secreted recombinant A-chain contains an additional alanine at the amino-terminus. The amino-terminus of ricin has prevously been shown to be mobile and flexible (Bushuev and Tonevitsky, 1989) and mutations within the first 18 residues (Bradley *et al.*, 1989) or a deletion of the first 9 residues of ricin A-chain (May *et al.*, 1989) do not alter activity or solubility. Based on the close structural relationship between ricin and abrin C an additional alanine residue at the amino terminus would not be expected to alter the properties of the protein significantly.

Periplasmic expression was successful, however because of the relatively low yield of A-chain obtained in the periplasmic fraction and the problems associated with scaling up the procedure for purification, cytoplasmic expression was pursued for purification of recombinant abrin A-chain.

3.8.2 Cytoplasmic expression

Cytoplasmic expression construct for native sequence

The first construct prepared for cytoplasmic expression consisted of the mature A-chain sequence cloned into pKK-233-2, utilising the ATG codon provided within the *NcoI* site of the vector. The mature A-chain sequence was recovered by PCR using the primers 9A-F and 9A-R (shown in Figure 28B). The PCR product was blunted at the 5' *SacI* site prior to digestion with *HindIII*. The A-chain fragment was then subcloned into the pKK233-2 vector which had been sequentially digested with *NcoI*, followed by filling in of the 5' overhang, and then *HindIII*. The final construct was designated as pKK-9A1. The cloning strategy is summarised in Figure 31.

Cytoplasmic expression in *E. coli*

The results from cytoplasmic expression of the mature A-chain at 37^{o}C using the construct pKK-9A1 are shown in Figure 32. On analysis by SDS-PAGE and Coomassie blue staining (Figure 32A) there is no evidence of an expressed protein of the expected size in the total cell fraction (lane 3) compared to the non-recombinant vector control (lane 2). Western blot analysis using [^{125}I]-protein A as the development system (Figure 32B) shows that there is an immunoreactive band of 28kDa present in the total cell sonicate (lane 3) which is not present in the control (lane 2). Recombinant A-chain is therefore being expressed but levels are exceedingly low at less than 0.1% of total protein. The product is also insoluble as the A-chain is found exclusively in the pelleted fraction (lane 4).

Figure 31 *Summary of the cloning strategy for cytoplasmic A-chain expression construct pKK-9A1*

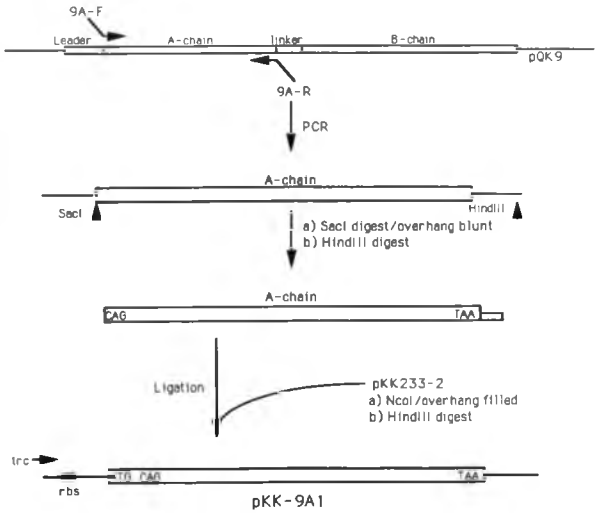
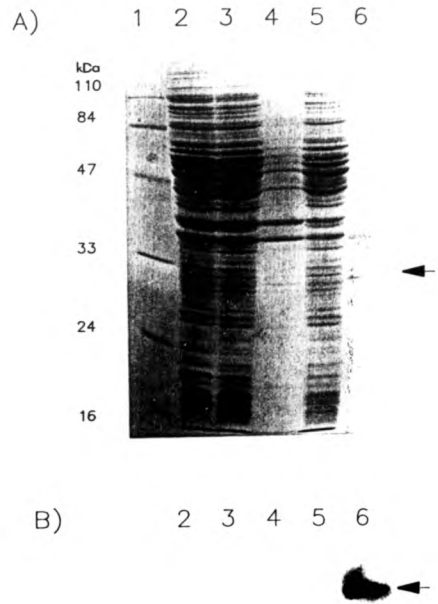


Figure 32 *Cytoplasmic expression of native A-chain sequence in E.coli* JM109 harbouring pKK-9A1 was cultured to an OD_{600} of 0.8, induced with 1mM IPTG and cultured for a further 3 hours. The cells were harvested and protein fractions were prepared as described (section 2.16.1). The protein fractions were analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the [^{125}I]-protein A development system as described (section 2.18.2). Lane 1, prestained low molecular weight markers; lane 2, non-recombinant vector control; lanes 3-5, total, insoluble and soluble fractions respectively from JM109/pKK-9A1; lane 6, native abrin C. The arrow indicates abrin C A-chain


A)
1 2 3 4 5 6
kDa
110
84
47
33
24
16
B)
2 3 4 5 6

Cytoplasmic expression of altered A-chain sequence

A potential reason for the low level of expression achieved with the construct pKK-9A1 may have been a consequence of secondary structure formation within the mRNA which might interfere with translation. On analysis of the mRNA that would be transcribed from the construct pKK-9A1, a hairpin loop structure, close to the start codon, which could form in the mRNA was noted. In an effort to improve levels of expression a second cytoplasmic expression construct was generated in which the codons at the 5' end of the mature A-chain sequence were altered to contain maximal A+T content and to reduce any potential secondary structure formation within the mRNA without altering the protein sequence.

A double stranded oligonucleotide encoding the 5' end of the A-chain sequence to a *HaeII* site at nucleotide position 68 was designed and is illustrated in Figure 33A. The oligonucleotides were designed so that, when annealed, the 5' end would consist of an *NcoI* overhang and the 3' end would consist of an *HaeII* overhang. The final construct designated pKK-9A2 was generated in a tripartite ligation containing the double-stranded oligonucleotide, the A-chain sequence isolated from pKK9A1 on a *HaeII* to *HindIII* fragment and the vector pKK233-2 digested with *NcoI* and *HindIII*. The cloning strategy is illustrated in Figure 33B.

The results from expression of the A-chain in *E. coli* at 37^{o}C using the construct pKK-9A2 is shown in Figure 34. Analysis by SDS-PAGE and Coomassie blue staining (Figure 34A) shows the presence of a band of 28kDa in the total cell fraction (lane 3) not visible in the non-recombinant vector control (lane 2). However the recombinant A-chain is found in the insoluble pellet fraction (lane 4). Insolubility of the expressed A-chain is confirmed by Western blot analysis using the [^{125}I]-protein A development system (Figure 34B) showing that the immunoreactive product of 28kDa is found exclusively within the insoluble fraction (lane 4).

Figure 33 *Replacement oligonucleotides and summary of the cloning strategy for cytoplasmic 5'-(A+T)-rich A-chain expression construct* The replacement oligonucleotide (A) is shown, with the A-chain coding sequence numbered for reference. The oligonucleotide was used to replace the 5' end of the A-chain sequence and the cloning strategy is summarised in (B)

A)

```
                                                                    22
   1
  Gln Asp Gln Val Ile Lys Phe Thr Thr Glu Gly Ala Thr Ser Gln Ser Tyr Lys Gln Phe Ile Glu
5'-CAA GAT CAA GTT ATT AAA TTC ACT ACT GAA GGA GCA ACT AGT CAA TCT TAT AAA CAA TTT ATT GAA GCG C-3'
3'-GTT CTA GTT CAA TAA TTT AAG TGA TGA CTT CCT CGT TGA TCA GTT AGA ATA TTT GTT AAA TAA CTT
                                                                    HaeII
```

B)

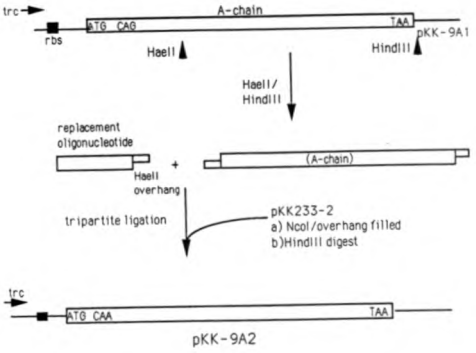
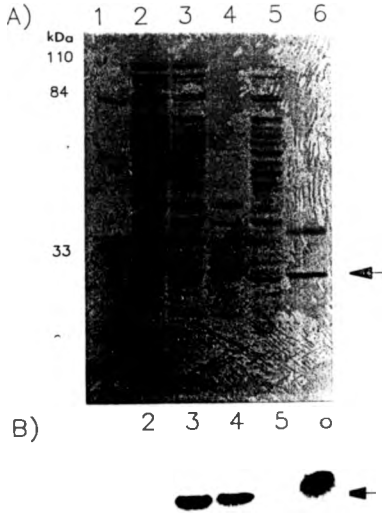


Figure 34 *Cytoplasmic expression of altered A-chain sequence in E. coli* JM109 harbouring pKK-9A2 was cultured to an OD_{600} of 0.8, induced with 1mM IPTG and cultured for a further 3 hours at 37°C. The cells were harvested and protein fractions were prepared as described (section 2.16.1). The protein fractions were analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the [^{125}I]-protein A development system as described (section 2.18.2). Lane 1, prestained low molecular weight markers; lane 2, non-recombinant vector control; lanes 3-5, total, insoluble and soluble fractions respectively from JM109/pKK-9A2; lane 6, native abrin C. The arrow indicates abrin C A-chain



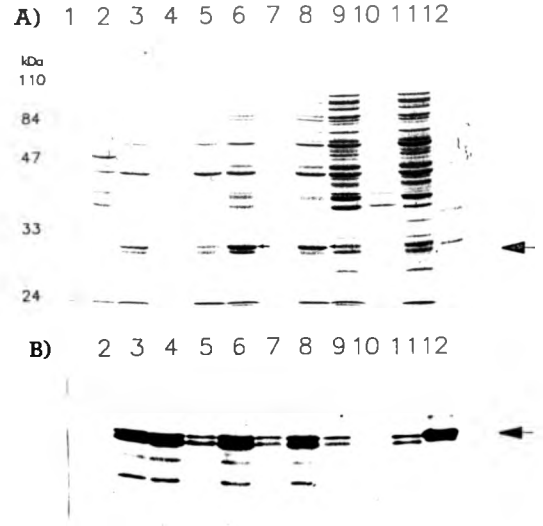
3.8.3 Effect of temperature on A-chain solubility

A comparison was made of the effect of temperature on A-chain expression: three cultures of JM109 harbouring the expression plasmid pKK-9A2 were grown at 37°C to an OD_{600} of 0.7, then two cultures were shifted to either 30°C or 18°C for 30 minutes, and at an OD_{600} of 0.8 all cultures were induced with IPTG.

The results of analysis by SDS-PAGE and Western blot are shown in Figure 35. On a Coomassie blue stained gel (Figure 35A) it is clear that at 30°C (lanes 6-8) the majority of the A-chain in the total cell fraction (lane 6) is found in the soluble fraction (lane 8). At 18°C there is an additional band in the total cell fraction (lane 9), which is also present in the soluble fraction (lane 11), compared to the control (lane 2). The Western blot (Figure 35B) confirms that the A-chain is soluble when expressed at 30°C. However the band of approximately 28kDa seen in the soluble fraction from expression at 18°C is not immunoreactive. It seems probable that these additional bands are *E. coli* host proteins which have been induced by the shift to the lower temperature.

Schein and Noteborn (1988) have shown that the formation of soluble recombinant proteins in *E. coli* is favoured by lower temperatures. Three different recombinant proteins (interferon-α-2, interferon-γ and murine protein Mx) with different physical properties undergo temperature dependent insolubilisation during growth in *E. coli* and the addition of soluble interferon- to *E. coli* lysates rendered the protein insoluble. It was postulated that the higher temperatures allow crossing of thermodynamic barriers to generate intermolecular reactions which involve *E. coli* host proteins. The rate of protein synthesis may also contribute to solubility since it is generally the overexpression of proteins that leads to insoluble aggregate formation (Kane and Hartley, 1988).

Figure 35 *Effect of temperature on solubility of expressed A-chain* Cultures of JM109 harbouring pKK-9A2 were induced at OD_{600} of 0.8 with 1mM IPTG and then cultured to an OD_{600} of 1.2 at either 37°C, 30°C or 18°C. Protein fractions were prepared and analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phophatase conjugate as described (section 2.18.2). Lane 1, prestained low molecular weight markers; lane 2, non-recombinant vector control; lane 3-5, total, insoluble and soluble fraction respectively at 37°C; lanes 6-8, total, insoluble and soluble fractions respectively at 30°C; lanes 9-11, total, insoluble and soluble fractions respectively at 18°C; lane 12, native abrin C. The arrow indicates the abrin A-chain



The expression of ricin A-chain in *E. coli* is found to be temperature dependent. Piatak *et al.*, (1988) showed that ricin A-chain was insoluble and inactive when expressed at 42°C and O'Hare *et al.* (1987) showed that ricin A-chain formed insoluble aggregates with reduced activity when expressed at 37°C compared to fully soluble, biologically active ricin A-chain expressed at 30°C. Trichosanthin, a type I ribosome-inactivating protein, undergoes a conformational change at 35°C (personal communication, Dr. J. Wang, Institute of Biophysics, Beijing, China). In nature, the ribosome-inactivating proteins would be expressed at temperatures considerably lower than 37°C and therefore the most energetically stable conformation of the protein i.e. a stable, soluble and active conformation, may be altered by the higher temperature leading to an insoluble protein of reduced or no activity

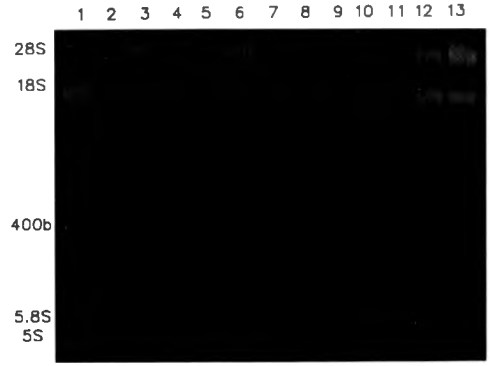
Analysis of the gel in Figure 35A, and others, using a densitometer shows that soluble A-chain is expressed at levels exceeding 6% of total cellular protein when expressed at 30°C.

The samples analysed by Western blot and shown in Figure 35B were 24 hours post-sonication and had been stored at 4°C. It is clear from the Western blot that there is breakdown of the A-chain into specific polypeptides on storage of the cell sonicates. Inclusion of leupeptin in sonication buffer did not improve on this degree of breakdown over a 24 hour storage time. Protease action on cytoplasmically-expressed heterologous proteins is a common problem (Marston, 1986).

3.8.4 *In vitro* activity in cell extracts containing A-chain

To determine activity of the expressed A-chain in the crude cell extracts, the *in vitro* assay was used (section 2.20.2). However the RNA was severely degraded due to RNases. To overcome this the crude cell extracts were preincubated with 20mM vanadyl ribonucleoside complex at 37°C for 1 hour prior to incubation with the ribosomes.

Figure 36 *N-glycosidase activity in crude extracts of E. coli expressing A-chain* The crude cell fractions were preincubated with 20mM vanadyl ribonucleoside complex for 1 hour at 30°C. A 30µl volume of rabbit reticulocyte lysate was incubated with 1µl of the crude cell extract for 30 minutes at 30°C; the RNA was extracted and treated with aniline for 2 minutes at 65°C and analysed as described (section 2.20.2). Lane 1, total extract from non-recombinant vector control; lanes 2-4, total, insoluble and soluble fractions respectively from JM109/pKK-9A2 cultured at 37°C; lanes 5-7, total, insoluble and soluble fractions respectively from JM109/pKK-9A2 cultured at 30°C; lanes 8-10, total, insoluble and soluble fractions respectively from JM109/pKK-9A2 cultured at 18°C; lane 11, total extract from JM109/pKK-9A2 cultured at 30°C, no aniline treatment; lanes 11 and 12, 0.1µg ricin A-chain plus and minus aniline treatment respectively. The diagnostic 400b fragment released from 28S rRNA by ricin is indicated



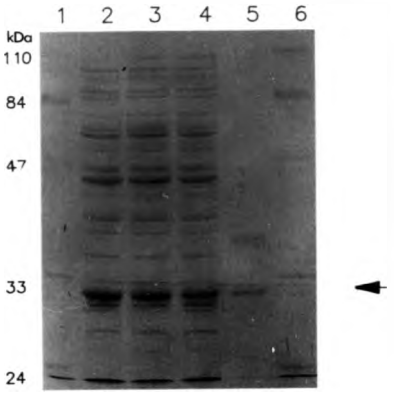
The results of the *in vitro* assay are shown in Figure 36. Lane 12 illustrates the diagnostic 400b fragment released from 28S rRNA by aniline after incubation with 0.1μg ricin. This 400b fragment is absent when treatment of aniline is omitted (lane 13) or incubation with ricin is omitted (lane 11). Lane 1 shows that there is no *N*-glycosidase activity associated with a non-recombinant vector control. Lanes 2 to 10 inclusive show that activity as a consequence of A-chain expression is found at all expression temperatures and activity can be detected in both soluble and insoluble fractions.

3.8.5 Effect of IPTG on A-chain expression levels

To determine if the amount of IPTG could alter or improve the amount of A-chain expressed several different IPTG concentrations were used for induction of A-chain expression at 30^{o}C. The result is shown in Figure 37. The amount of soluble A-chain produced is not significantly altered by the IPTG concentration but 1mM IPTG was the optimum of the concentrations tested here (lane 1).

Previously very low concentrations of IPTG (5μM) for induction have been shown to improve the production of heterologous genes expressed in *E. coli* (Takagi *et al.*, 1988). Generally however the low inducer concentrations improve expression levels when higher IPTG concentrations lead to a massive induction of the heterologous protein which forms aggregates. By slowing down the rate of expression and preventing aggregate formation, the amount of soluble product is increased. In the system used here the product is not forming aggregates and a slower rate of production of the A-chain does not improve the yield.

Figure 37 *Effect of IPTG concentration, at induction, on A-chain expression* Cultures of JM109 harbouring pKK-9A2 were induced at OD_{600} of 0.8 and at 30°C with either 1mM, 100μM or 100nM IPTG. Total cell sonicates were analysed by SDS-PAGE and Coomassie blue staining. Lanes 1 and 6, prestained low molecular weight markers; lane 2, 1mM IPTG; lane 3, 100μM IPTG; lane 4, 100nM IPTG; lane 5, native abrin C. The arrow indicates the A-chain



3.8.6 Growth rates and plasmid stability

E. coli ribosomes have been shown to be resistant to the action of ricin and abrin C therefore toxicity of the recombinant protein to the cells was not expected. However, during the construction of the vectors pKK-9A1 and pKK-9A2, no transformants could be obtained when the host strain used to prepare competent cells was JM109 which had lost the F' episome. The episome carries the gene for the repressor of the *tac* promoter such that there is no transcription in the absence of inducer. The problem of low transformation efficiency in JM109/F'$^-$ suggested that expression of the recombinant A-chain was leading to negative selection pressure.

Habuka *et al.*, (1990) have shown that a type I ribosome-inactivating protein, *Mirabilis* antiviral protein (MAP), inhibited the growth of *E. coli* when (MAP) was expressed cytoplasmically. MAP was found to have an IC_{50} of 200nM in an *E. coli in vitro* translation system. Habuka *et al.* (1989) could not obtain transformants containing the MAP expression plasmid in strains lacking *lacI*^{q} such as HB101 and DH1. Trichosanthin has been expressed cytoplasmically in *E. coli*, (Chow *et al.*, 1990), and constitutive expression during growth of the host strain gave optimal levels of the recombinant trichosanthin (personal communication, Dr M. Piatak, Genelabs, California, USA). Chow *et al.* did not analyse the *E. coli* ribosomes, from a culture expressing trichosanthin, for modification but based on the good growth of the cultures and the ability of the host to support constitutive expression of trichosanthin it is highly improbable that trichosanthin modifies *E. coli* ribosomes.

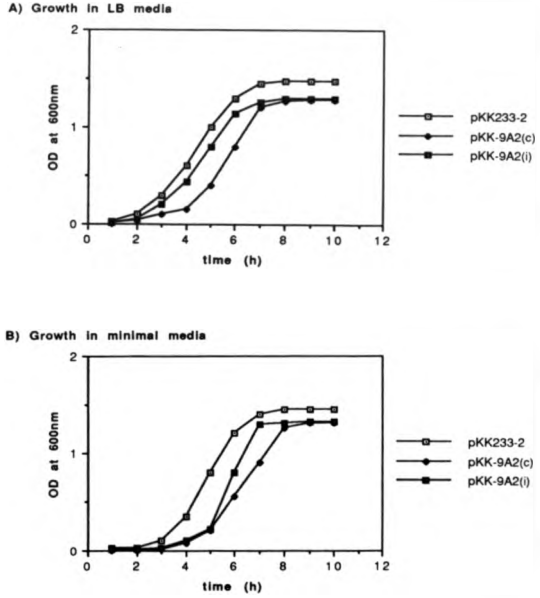
Faced with the possibility that the recombinant abrin was toxic to *E. coli*, transformations were carried out using minimal media to prepare competent cells and to plate out transformants. This was to ensure maintenance of the F' episome to prevent expression of the recombinant abrin in positive transformants. Percent positive transformants in JM109 carrying the episome was high using this method. It should be noted that

in generating the periplasmic constructs no problems with obtaining positive transformants occurred. In the case of periplasmic expression the protein is expected to be exported to the periplasmic space, and therefore the recombinant abrin will be sequestered in a compartment without access to the host ribosomes. In addition, although ricin A-chain retains activity with an amino-terminal extension, in the form of a fusion protein (Frankel *et al.*, 1989 and O'Hare *et al.*, 1987), the Omp A signal peptide may alter the conformation of the protein sufficiently to alter activity.

Growth curves of various cultures of JM109 harbouring pKK-9A2 under different conditions of induction and in either minimal media or LB media are shown in Figure 38A and Figure 38B respectively. The optical density of the control (JM109/non-recombinant vector control) was always higher after extended periods of growth than a recombinant vector. This suggested that the promoter in the expression constructs is leaky and even low levels of expression slowed growth. Recombinant A-chain could be detected in cultures grown in LB and minimal media, in the absence of added IPTG although in minimal media the level of expressed A-chain was at the lower limit of detection. Growth is slowed by constitutive expression of recombinant A-chain in both LB and minimal media and the yield of A-chain was less than from cultures induced during late exponential growth.

The degree of inhibition of growth due to expression of the recombinant abrin A-chain was significantly less than that seen for MAP where growth of the *E. coli* host stopped within 1 hour of expression. It therefore seemed most likely that it was the burden on the host cell of producing the additional recombinant protein and of replicating the larger plasmid that was slowing growth rather than any toxicity of the protein *per se*.

Figure 38 *Growth curves of JM109 harbouring pKK-9A2* Overnight cultures of JM109 harbouring pKK9A2 or pKK233-2 in minimal media supplemented with ampicillin were diluted 1 in 100 into either (A) LB media, or (B) minimal media and were cultured at 30°C. The cultures were induced at either t=0 (c) or when the OD_{600} reached 0.8 (i).



Minimal media was selected for expression of recombinant A-chain for purification, and the cultures were induced at the end of exponential growth when the cells would be able to support the rapid burst of protein synthesis. A supplement of caseamino acids provided an exogenous source of amino acids at this stage.

Harris and Emtage (1986) reported the instability of a plasmid encoding prochymosin during culture. To check the stability of the plasmid and the F' episome in the system described here aliquots of a culture of JM109/pKK-9A2 grown in minimal media to OD_{600} of 0.8, then induced with 1mM IPTG and cultured for a further 3 hours, were serially diluted and plated out on several different selection plates in triplicate: LB plates (for total counts), LB plates supplemented with ampicillin (for cells retaining the plasmid), minimal media plates (for cells retaining the episome) and minimal media plates supplemented with ampicillin (for cells retaining both plasmid and episome). The results (Table 5) showed that the difference in number of cells on each of the four selection plates was not statistically significant with the range being from 95% to 102% of the control. This showed that under the conditions of expression used the plasmid and the episome were stable.

Table 5 *Stability of pKK-9A2 and the F' episome in JM109* A culture of JM109 harbouring pKK-9A2 was cultured in minimal media to an OD_{600} of 0.8, induced with 1mM IPTG and supplemented with 2% caseamino acids then cultured for a further 3 hours. The culture was serially diluted and plated out in triplicate onto selective media plates. The experiment was carried out in triplicate

Selective media	Number of colonies as % of control			
	1	2	3	Average
LB	100	100	100	100
LB + ampicillin	96	108	102	102
Minimal media	88	84	113	95
Minimal media + ampicillin	102	101	94	99

3.8.7 Summary of A-chain expression

To obtain satisfactory amounts of recombinant A-chain for purification it was necessary to alter the nucleotide sequence of the A-chain to reduce secondary structure within the transcribed RNA. Cytoplasmic expression of soluble active A-chain at levels exceeding 6% of total protein were achieved by reducing the temperature of the cultures at induction, with 1mM IPTG, to 30^{o}C. Constitutive expression of the A-chain did not improve yield. Minimal media, supplemented with 2% caseamino acids at induction, gave the optimum yield of A-chain and under the selected conditions for large scale culture the plasmid and F' episome are stable.

A problem encountered with cytoplasmic expression was that of protease action on the recombinant abrin A-chain. The degree of breakdown increased with storage time. The amount of intact A-chain even after 24 hours of storage was significantly higher than for the other constructs and with prompt purification the degree of breakdown could be minimised.

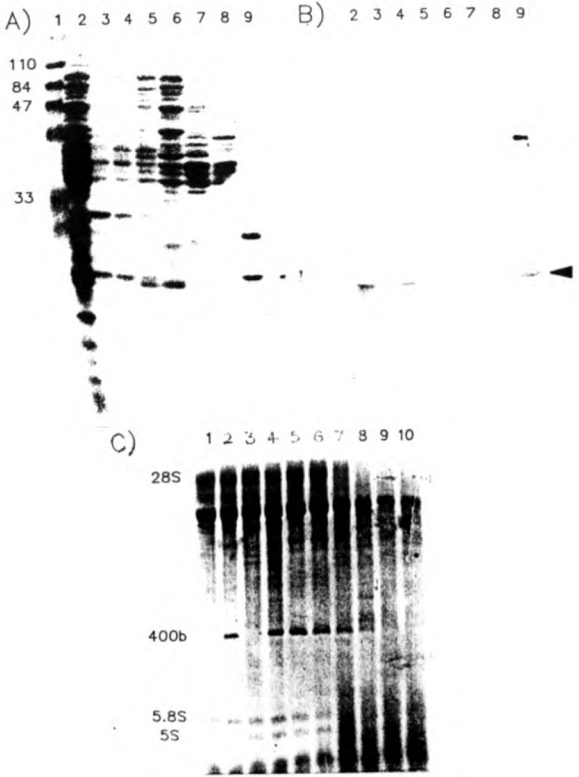
3.9 PURIFICATION OF RECOMBINANT A-CHAIN

3.9.1 Ammonium sulphate precipitation

Initially a 50% ammonium sulphate precipitation was used to reduce the volume of crude cell extract for ease of handling. At 50% all the A-chain precipitated. However when a range of ammonium sulphate concentrations were used sequentially to precipitate proteins it was found that intact A-chain precipitated at lower ammonium sulphate concentrations than the degraded A-chain fragments. The result is shown in Figure 39. This procedure allowed removal of the smaller A-chain products and also allowed analysis of the breakdown polypeptides in isolation. For the final scheme devised for large scale purification of A-chain a 20-30% ammonium sulphate cut was used to concentrate and enrich for the A-chain.

Figure 39 *Ammonium sulphate precipitation of cell sonicates* Solid ammonium sulphate was added to crude cell sonicate and the precipitated proteins were recovered by centrifugation and resuspended in 1 times SDS-PAGE loading buffer. Sequential ammonium sulphate precipitations of increasing concentrations were performed on the supernatant. The samples were analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (section 2.18.2). Lane 1, prestained low molecular weight markers; lane 2, total cell sonicate; lanes 3-8, 0-20%, >20%-30%, >30%-40%, >40%-50%, >50%-60% and >60%-70% ammonium sulphate precipitations respectively; lane 9, native abrin C. The arrow indicates the A-chain.

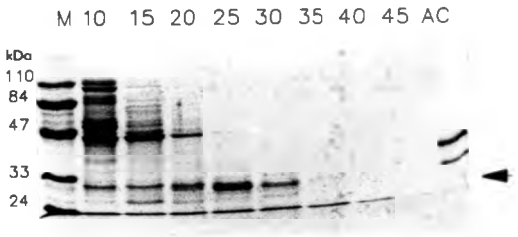
(C) *N-glycosidase activity in precipitated fractions* Each sample was assayed for *in vitro* activity as described in the legend to Figure 36. Lanes 1 and 2, 1µg ricin A-chain minus and plus aniline treatment respectively; lanes 4 and 5, total cell sonicate minus and plus aniline treatment respectively; lanes 5-10, as for lanes 3-8 above respectively


A) 1 2 3 4 5 6 7 8 9
B) 2 3 4 5 6 7 8 9
110
84
47
33
C) 1 2 3 4 5 6 7 8 9 10
28S
400b
5.8S
5S

3.9.2 Gel filtration on Sephadex G75

To reduce the amounts of contaminating *E. coli* proteins from the ammonium sulphate precipitation, gel filtration on G75 was used. The result is shown in Figure 40. Gel filtration gave a reasonable enrichment of A-chain when a small volume column was used (1cm x 30cm) and analytical ammounts were loaded. However when the procedure was scaled up to a 1m x 2.5cm column the results were less satisfactory and the A-chain eluted within a large volume which would therefore require concentration before application to the next step in purification. Gel filtration was therefore not used in purification of A-chain from a 1 litre culture.

Figure 40 *Gel filtration on Sephadex G-75* A 20%-30% ammonium sulphate precipitate was resuspended in 1/10 growth volume of 10mM phosphate buffer, pH 7.5, 250mM NaCl and then dialysed against this buffer overnight at 4°C. A 0.5ml volume was loaded onto a 1 x 30cm Sephadex G-75 column pre-equilibrated with 10mM phoshate buffer, pH 7.5, 250mM NaCl. The column was washed with 10mM phosphate buffer, pH 7.5, 250mM NaCl at a flow rate of 0.25ml per minute and 1ml fractions were collected. The fractions were analysed by SDS-PAGE and Coomassie blue staining. M, prestained molecular weight markers; AC, native abrin C. The fraction numbers analysed are indicated and the arrow indicates the A-chain



3.9.3 HPLC on DEAE-Sephadex

Because of the problem of breakdown of the intact A-chain by *E. coli* proteases a purification step which gave a high degree of enrichment of the A-chain and removal of the contaminating proteases was required. In addition the conditions must be non-denaturing. Sephadex was the matrix of choice because it is bead-formed and therefore has good flow properties and packs easily. Sephadex is hydrophilic and therefore has low non-specific absorbance for proteins and does not denature biological molecules. To select the functional group, small 1ml columns of several ion exchangers were prepared in 1ml disposable syringes to analyse for A-chain binding. DEAE-Sephadex was shown to be suitable and was selected for large scale purification of the recombinant abrin A-chain.

The elution profile from HPLC using the DEAE-Sephadex column is shown in Figure 41. The A-chain eluted at approximately 250mM NaCl and was readily detected on stained SDS-PAGE gel (Figure 41A) in a single peak which was generally found in two sequential 6ml fractions. This was confirmed by Western blot analysis (Figure 41B). Once the elution profile of the A-chain had been determined, 1ml fractions were collected during the portion of the gradient when the A-chain eluted and Figure 42A shows a stained gel of sequential fractions containing the A-chain eluted at 250mM NaCl from a DEAE-Sephadex column. A high degree of enrichment was obtained and the majority of contaminating proteins were removed in this single stage of purification. A range of pH conditions was analysed but no improvement on the degree of purification was obtained. At pH9 or above, the capacity of the column was reduced significantly and at pH 7 the A-chain eluted at a lower NaCl concentration with an increase in the amounts of contaminating protein.

Figure 41 *Enrichment of the A-chain by HPLC on DEAE-Sephadex* A 20%-30% ammonium sulphate precipitation of cell sonicate was resuspended in 1/10 growth volume 10mM phosphate buffer, pH 7.5, 25mM NaCl (start buffer). The sample was dialysed against the start buffer overnight at $4^{o}C$. A DEAE-Sephadex column was equilibrated with start buffer at a flow rate of 3ml per minute and the OD_{280} baseline was established. A 5ml sample was loaded and the column was washed with start buffer until the baseline value was reached. Bound proteins were eluted with a gradient of NaCl from 25mM to 500mM in 10mM phosphate buffer, pH 7.5 at a flow rate of 3ml per minute and 6ml fractions were collected. The eluted fractions were analysed by (A) SDS-PAGE and Coomassie blue staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (section 2.18.2). M, prestained molecular weight markers; AC, native abrin C; S, loaded sample. The fraction numbers analysed are indicated and the arrow indicates the A-chain

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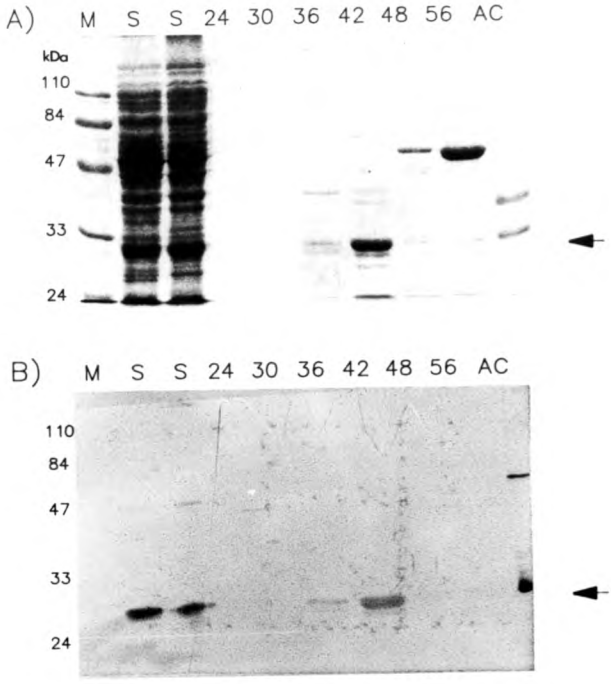
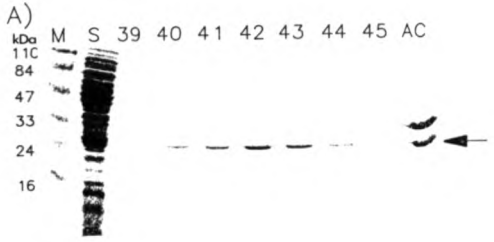
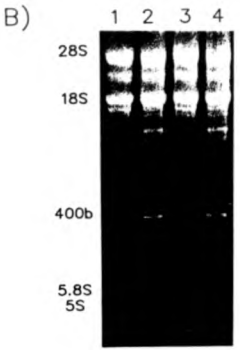

A)
M S S 24 30 36 42 48 56 AC
kDa
110
84
47
33
24
B)
M S S 24 30 36 42 48 56 AC
110
84
47
33
24

Figure 42 *Activity of the A-chain-containing fractions eluted from DEAE-Sephadex* HPLC on DEAE-Sephadex was performed as described in the legend to Figure 41 except that 1ml fractions were collected. The fractions containing the A-chain are shown by (A) SDS-PAGE and Coomassie blue staining where the fraction numbers analysed are indicated. M, prestained molecular weight markers; AC, native abrin C. The arrow indicates the A-chain. The fraction numbers 39-45 shown in (A) were pooled and a 1ul aliquot was used to (B) determine the activity against rabbit reticulocyte lysate as described (section 2.20.2). Lanes 1 and 2, treatment with 0.1ug ricin A-chain minus and plus aniline respectively; lanes 3 and 4, 1ul partially purified abrin A-chain minus and plus aniline treatment respectively


A)
kDa
M S 39 40 41 42 43 44 45 AC
110
84
47
33
24
16

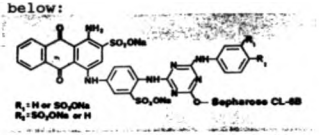

B)
1 2 3 4
28S
18S
400b
5.8S
5S

The fractions containing A-chain were pooled and a 1µl aliquot was used to determine if *in vitro* activity could be detected against rabbit reticulocyte lysate (section 2.20.2). The result is shown in Figure 42B. The diagnostic 400b fragment is clearly visible when the ribosomes were incubated with the pertially purified A-chain followed by aniline treatment of the rRNA (lane 4).

The intact A-chain was contained in a total of less than 10ml eluent, which allowed direct application to the Blue-Sepharose CL-6B column, after dialysis, without concentration.

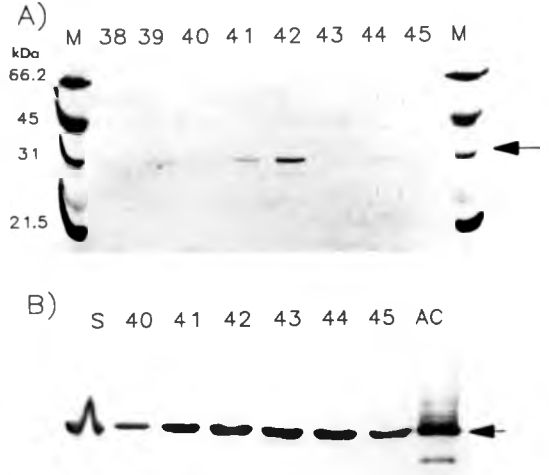
2.9.4 Purification on Blue-Sepharose CL-6B

For the final step in purification affinity chromatography was chosen to select the recombinant abrin A-chain on the basis of its biological function. Blue Sepharose CL-6B contains the dye, Cibacron Blue F3G-A, which has affinity for a variety of enzymes and proteins. The partial structure of Blue Sepharose CL-6B is shown below:



Ricin binds to Cibacron Blue (Appukuttan and Bachhawat, 1979) as does gelonin and it has been postulated that the interaction of the ribosome-inactivating proteins with the dye is via the active site of these proteins. The dye resembles dinucleotides in 3-dimensional structures (Knowles and Thorpe, 1987) and it can be envisaged that the ribosome-inactivating proteins can interact with the ligand in a similar manner to the way they would interact with the RNA in the ribosome. Blue-Sepharose has been used to purify immunotoxins containing ricin A-chain, abrin A-chain and gelonin from unconjugated antibody (Lambert *et al.*, 1985, Cumber *et al.*, 1990 and Ghetie *et al.*, 1990).

Figure 43 *Purification of A-chain on Blue-Sepharose CL-6B* The partially purified A-chain was dialysed against 10mM phosphate buffer, pH 7.5, 25mM NaCl overnight at 4°C. A 1 x 30cm column of Blue-Sepharose CL-6B was eqilibrated with 10mM phosphate buffer, pH 7.5, 25mM NaCl at a flow rate of 0.2ml per minute and the OD_{280} baseline was established. The sample was loaded and the column was washed with 10mM phosphate buffer, pH 7.5, 25mM NaCl at a flow rate of 0.125ml per minute until the baseline value was reached. Bound protein was eluted with a gradient of NaCl from 25mM-1000mM in 10mM phosphate buffer, pH 7.5, at a flow rate of 0.125ml per minute and 1ml fractions were collected. The fractions were analysed by (A) SDS-PAGE and silver staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (section 2.18.2). M, prestained molecular weight markers; AC, native abrin C. The fraction numbers analysed are indicated and the arrow indicates the A-chain



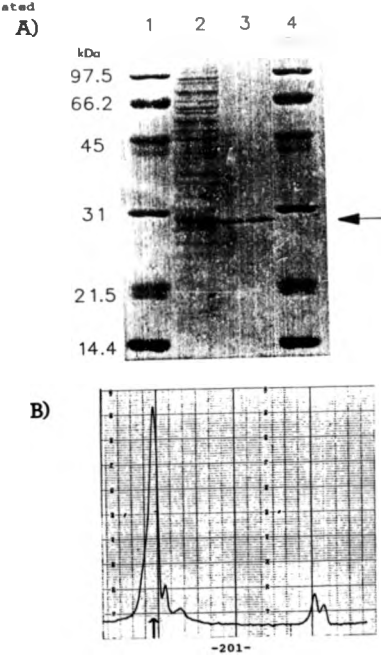
The elution profile obtained from Blue-Sepharose CL-6B is shown in Figure 43. The A-chain eluted as a single peak in approximately 5ml of eluent at 300mM NaCl.

The pooled A-chain fractions were analysed by SDS-PAGE and by HPLC on DEAE-Sephadex and the results are shown in Figure 44. The A-chain is estimated to be greater than 99% pure. Using the extinction coefficient of abrin C A-chain of 7.87 the yield of pure A-chain was approximately 1mg/ litre of culture.

2.9.5 Storage of purified A-chain

Ricin A-chain and the type I ribosome-inactivating proteins are generally stored at 4^{o}C without significant loss of activity. The purified abrin-related A-chain was initially stored at 4^{o}C but was found to lose all activity within 14 days. The protein also insolubilised during this time. The isoform abrin-b was also noted to be relatively unstable (Lin *et al.*, 1982). The A-chain was therefore stored at -20^{o}C in the presence of 15% (v/v) glycerol. The A-chain was stable under these conditions as determined from the *in vitro* assay for activity (section 2.20.2).

Figure 44 *Analysis of the purity of the A-chain* The fractions containing A-chain that were eluted from Blue-Sepharose CL-6B were pooled and dialysed against PBS overnight at 4°C. The purified A-chain was analysed by (A) SDS-PAGE and Coomassie blue staining; lanes 1 and 4, molecular weight markers; lane 2, 0.01 OD_{280} units cell sonicate; lane 3, 0.01 OD_{280} units purified A-chain; and by (B) HPLC on DEAE-Sephadex as described (section 2.19.3). The peak corresponding to the A-chain is indicated



3.10 *IN VITRO* ACTIVITY OF PURIFIED RECOMBINANT A-CHAIN

3.10.1 Sensitivity of ribosomes to A-chain

To determine the sensitivity of ribosomes to the activity of the purified recombinant A-chain, ribosomes from a variety of sources were used as the substrate in the *in vitro* assay (section 2.20.2). The positive control used was incubation of the ribosomes with 0.1μg PAP which has been shown to be active on all the ribosome species analysed here (personal communication, Dr M.R. Hartley, University of Warwick).

The result is shown in Figure 45. The diagnostic fragment is absent when aniline treatment is omitted (lanes 1 and 3) or without treatment of the ribosomes with a ribosome-inactivating protein (lane 1). The A-chain has activity against yeast ribosomes (Y, lane 3), rabbit reticulocyte lysate ribosomes (R, lane 3) and tobacco ribosomes (T, lane 3) but no activity was detected against chloroplast ribosomes (C, lane 3) or *E. coli* ribosomes (E, lane 3). The concentration of the recombinant A-chain in this assay is approximately 6×10^{-6}M.

The type II ribosome-inactivating proteins abrin C and ricin share a similar profile of activity: eukaryotic ribosomes are sensitive whereas prokaryotic ribosomes are insensitive.

Plant ribosomes tend to be less sensitive to the action of ribosome-inactivating proteins. Stirpe and Hughes (1989) determined the IC_{50} values of 6 different ribosome-inactivating proteins against a variety of plant and protazoan species using poly(U)-directed phenylalanine polymerisation. The results showed that ribosome-inactivating proteins were inactive against ribosomes from their own plant species but ribosomes from any one plant showed a large range of sensitivities to different ribosome-inactivating proteins. Ribosome-inactivating proteins from taxonomically related plants were more similar, in their activity, to each other than to ribosome-inactivating proteins from distantly-related species.

Figure 45 *Sensitivity of ribosomes to the A-chain* Approximately 10μg of ribosomes, isolated from *S.cerevisiae* (Y), rabbit liver (R), tobacco leaves (T), spinach chloroplasts (C) or *E. coli* (E), were incubated with either 0.1μg PAP or 1μg purified recombinant abrin A-chain for 1 hour at 30°C. The rRNA was extracted and incubated with aniline and analysed as described (section 2.20.2). Lanes 1, no treatment; lane 2, incubation with PAP; lane 3, incubation with the abrin A-chain; lane 4, incubation with the abrin A-chain minus aniline treatment The diagnostic fragment released by PAP is indicated

Unfortunately, Stirpe does not indicate how the plant ribosomes were isolated and therefore the ribosome preparations may have been heavily contaminated with chloroplast ribosomes. Chloroplast ribosomes were shown here to be resistant to the action of the recombinant abrin A-chain and chloroplast ribosomes are insensitive to ricin A-chain (personal communication, Dr M.R. Hartley, University of Warwick). The detection method used of poly(U)-directed phenylalanine polymerisation is a relatively poor method for determining activity therefore the results of Stirpe and co-workers must be viewed with caution.

It is clear that there is extensive variability in the sensitivity of ribosomes to the action of ribosome-inactivating proteins. Amongst the protozoa, *Tetrahymena pyriformis* and Acanthamoeba castellanii ribosomes show differing degrees of sensitivities to dianthin, abrin and ricin (Cenini *et al.*, 1987). Brigotti *et al.* (1989) has shown that this variability extends to the metazoa. *Artemia salina* ribosomes are sensitive to PAP, ricin and momordin but are remarkably resistant to gelonin. The ribosomes of the *Trypanosoma* and of the *Leishmania* exibit different sensitivities to abrin but are resistant to ricin (Cenini *et al.*, 1988).

Generally, ribosomes are resistant to the action of the ribosome-inactivating protein(s) from the parent plant but this is not absolute. Clearly, if the plant is susceptible to the action of its own ribosome-inactivating protein the protein must be sequestered within a compartment of the plant cell or maintained in an inactive conformation. The spacial distribution of most ribosome-inactivating proteins is not known. PAP has been shown to be localised within the cell wall matrix of mesodermal cells of *P. americana* and is therefore extracellular (Ready *et al.*, 1986). Taylor and Irvin (1990) have shown that ribosomes from *P. americana* are depurinated during extraction. Ricin and abrin are sequestered within the protein storage bodies and until sequestration are in an inactive conformation due to the presence of the linker

sequence. Ricin A-chain has recently been shown to be active against *R. communis* ribosomes but the ribosomal RNA in the seed remains unmodified (Dr. M.R. Hartley, unpublished data).

The popular theory for the function of these ribosome-inactivating proteins as anti-viral agents relies on the host plant being susceptible to the action of its own ribosome-inactivating protein: on damage to the host cell by e.g. an aphid, or grazing of the leaf by herbivores, the ribosome-inactivating protein is released from its storage compartment and kills the cell. Once the cell wall has been compromised the cell is open to attack by virus and the ribosome-inactivating proteins may act as a host suicide agent preventing viral spread from the primary site of infection.

Recently, three proteins isolated from barley seeds were found to act synergistically in inhibiting the growth of fungi. The three proteins were a chitinase, a (1-3)-ß-glucanase and a 30kDa ribosome-inactivating protein. The action of the ribosome-inactivating protein was enhanced by permeabilisation of the hyphal walls by the action of the chitinase and glucanase. There may therefore be other proteins present in the tissue containing the ribosome-inactivating protein which act synergistically in inhibiting the growth of fungi.

3.10.2 Quantitative analysis of A-chain activity

The activity of the recombinant A-chain against yeast and rabbit reticulocyte lysate ribosomes was quantitated by using ten-fold serial dilutions of the A-chain in the assay. The results are shown in Figure 46. The lowest concentration of A-chain which modified rabbit reticulocyte lysate ribosomes was 6×10^{-14}M (Figure 46A) and the lowest concentration of A-chain which modified yeast ribosomes was 6×10^{-12}M (Figure 46B). The values obtained are 10-100 fold higher than those obtained for recombinant ricin A-chain assayed under the same conditions (Osborn, 1991). A direct comparison was not performed because the recombinant ricin A-chain available for assay had been

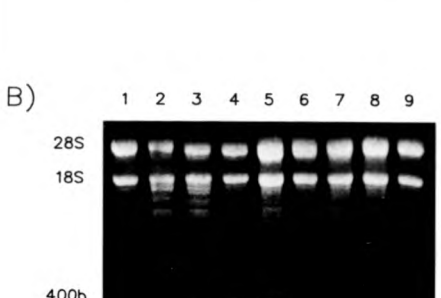
stored for several months and activity was therefore lower than freshly purified recombinant A-chain.

The assay used here to determine the activity of the recombinant abrin A-chain is not ideal but provides an end point below which activity is not detected. The degree of modification prior to the end point can be determined by scanning densitometry analysis of the gels following electrophoresis of the modified rRNA.

Figure 46 *Quantitative analysis of the activity of the A-chain* Serial dilutions of the purified recombinant A-chain were incubated with 10μg salt washed ribosomes for 1 hour at 30°C. The rRNA was extracted and treated with aniline and analysed as described (section 2.20.2). (A) Rabbit reticulocyte lysate ribosome incubated with: lane 1, PBS/aniline; lanes 2 and 3; 0.1μg ricin A-chain, minus and plus aniline treatment repectively; lanes 4 and 5, 0.1μg abrin A-chain, minus and plus aniline respectively; lanes 6-12, 10pg, 1pg, 0.1pg, 0.01pg, 1fg, 0.1fg and 0.01fg Abrin A-chain, plus aniline, respectively. (B) *S. cerevisiae* ribosomes treated with : lane 1, PBS/aniline; lanes 2 and 3, 0.1μg ricin A-chain minus and plus aniline respectively; lanes 4-9, 0.1 μg, 10ng, 1ng, 100pg, 10pg and 1pg abrin A-chain, plus aniline, respectively

-facing 207-


A)
1 2 3 4 5 6 7 8 9 10 11 12
28S
18S
400b
5.8S
5S


B)
1 2 3 4 5 6 7 8 9
28S
18S
400b
5.8S
5S

The other assays commonly used include inhibition of protein synthesis in cell free systems (Olsnes and Pihl, 1982) and the translation of a second message in the presence of a ribosome-inactivating protein mRNA (May *et al.*, 1989). Neither of these methods allow true quantitation of the activity of ribosome-inactivating proteins.

A new method was developed by Zamboni *et al.* (1989) to measure the amount of adenine released from ribosomes on treatment with a ribosome-inactivating protein and is based on the conversion of adenine to 1,N^6-ethano derivatives and quantitative detection using HPLC. Using [^{14}C]-labelled adenine, no exchange or reverse reactions were detected and the product did not inhibit depurination.

The method of Zamboni *et al.* allowed the effect of ricin on ribosomes which had been pretreated with a-sarcin to be determined. It had been shown previously that ricin sensitises ribosomes to the activity of a-sarcin (Terao *et al.*, 1988). Pretreatment of ribosomes with a-sarcin significantly reduced the *N*-glycosidase action of ricin (Sperti *et al.*, 1989), and the conformational changes induced in the L3-L4 domain by a-sarcin were postulated to be responsible. From the results of Sperti *et al.* it was evident that the amount of adenine released did not correlate with the inhibition of protein synthesis. The maximum amount of adenine released was 0.8 mole per mole of ribosomes. At 2/3 of the maximum amount of adenine released protein synthesis was inhibited by 90%, at 1/3 maximum adenine release protein synthesis was inhibited by 66%. A possible conclusion which might be drawn from these results is that only a proportion of ribosomes are sensitive to the action of ricin, equivalent to about 20% of the total population. In addition only a proportion of the ribosome population may be involved in active protein synthesis. Nygard and Nillson (1988) demonstrated that about 80% of the ribosome population formed a stable high-affinity complex with EF-2 in the presence of GTP, the remainder formed a low-affinity complex. Treatment with ricin A-chain generated a homogeneous population with the low-

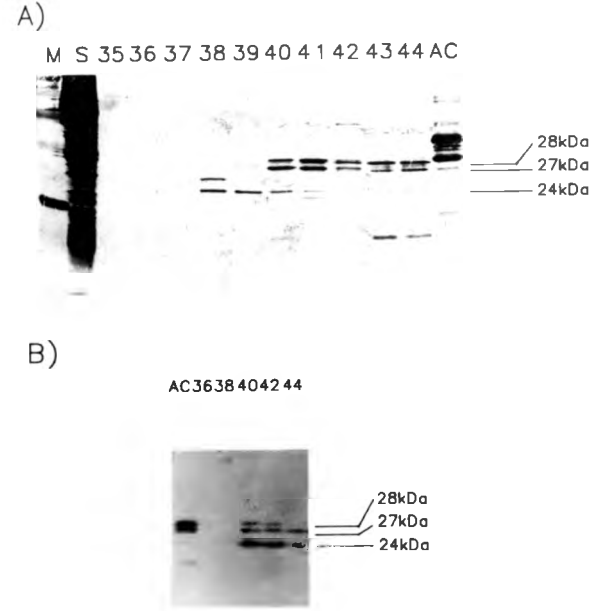
affinity binding properties. The results of Zamboni *et al.* (1989) support the notion that modification of a ribosome bound to mRNA inactivates the polysome, the majority of the ribosomes being protected from the action of ricin A-chain by bound EF-2.

The development of a truly quantitative and rapid assay will be complex, requiring consideration of a potentially resistant ribosome population, of inactivation of polysomes by the modification of only one or a few molecules of 28S rRNA within the polysome and of the protective effect of EF-2

The specific depurination reaction carried out by the ribosome-inactivating proteins is now well understood but the gross effects on the protein synthesising machinery continues to be investigated. It now seems clear that initiation is inhibited by these proteins in addition to elongation. Osborn and Hartley (1990) followed the appearance of [^{35}S]-methionine from initiator [^{35}S]-Met tRNA into 40S subunits, 80S monosomes and 80S polysomes in the presence and absence of ricin A-chain. Accumulation of [^{35}S]-methionine in monosomes which did not enter polysomes was noted. The formation of the 80S complex was reduced 6-fold with ricin A-chain compared to diphtheria toxin. Inhibition of translocation was implicated by the predominance of Met-Val when the system was programmed with globin mRNA. Puramycin had no effect on this system indicating that the acceptor site was occupied.

Together, the experiments on the effect of the ribosome-inactivating proteins, suggest that the modification to the 28S rRNA leads to a conformational change in the structural integrity of the ribosome (Endo *et al.*, 1988). This alteration slows the formation of 80S monosomes, and therefore of polysomes (Osborn and Hartley, 1990). The binding of EF-2 is prevented by the modification (Nilsson and Nygard, 1986) and the GTPase activity complemented by both EF-1 and EF-2 is inhibited (Benson *et al.*, 1975 and Sperti *et al.*, 1975) although the direct binding of EF-1 or the EF-1-dependant binding of amino-acyl tRNAs is unaltered (Montanaro *et al.*, 1973, Nilsson and Nygard, 1986 and Nilsson *et al.*, 1986).

Figure 47 *Binding of the A-chain breakdown products to DEAE-Sephadex* A 50% ammonium sulphate precipitation of a total cell sonicate was analysed by HPLC on DEAE-Sephadex as described (section 2.19.3). Sequential fractions were analysed by (A) SDS-PAGE and silver staining and by (B) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (2.18.2). M, prestained protein standards; AC native abrin C; S, sample analysed. The fraction numbers analysed are indicated and the intact A-chain and the major breakdown products are marked



3.10.10 Properties of the breakdown products of the A-chain

Figure 47A shows that the breakdown products elute from DEAE-Sephadex with a very similar profile to the intact A-chain when a 50% ammonium sulphate precipitation was used to concentrate the cell sonicate. The two major breakdown products are approximately 27kDa and 24kDa and may consist of the A-chain minus the amino-terminus and/or the carboxy-terminus. The reduction in size is not sufficient to alter the charge on the proteins at pH 8.5. The Western blot shown in Figure 47B illustrates the breakdown products more clearly.

Ammonium sulphate precipitation, to separate the breakdown products from the intact A-chain, provided the opportunity to study these proteins in isolation. A 35-50% ammonium sulphate precipitation was used to isolate the breakdown products from intact A-chain prior to application to DEAE-sephadex. The immunoreactive A-chain-derived polypeptides eluted from DEAE-Sephadex are shown in Figure 48A. Lane 3 of Figure 48A shows the pooled fractions containing the A-chain breakdown products. Intact A-chain in this fraction was below the limit of detection by Western blot. The fraction illustrated in lane 3, Figure 48A, was analysed for activity, and as shown in Figure 48B, modification to rRNA was detected and therefore at least one of the breakdown products visualised in Figure 48A, lane 3, retains *N*-glycosidase activity.

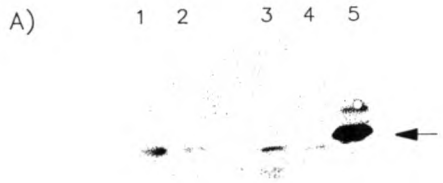
The properties of the two major breakdown products was different on Blue-Sepharose CL-6B. The larger of the two proteins bound to the column and eluted at 300mM NaCl and therefore behaved as the intact A-chain under the conditions used. The smaller of the two proteins did not bind to the column and was eluted in the void volume. This indicated that there had been a significant change in the properties of this peptide compared to the intact A-chain.

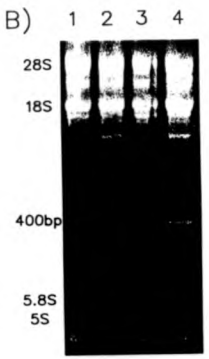
Figure 48 *The properties of the 27kDa breakdown product* A 35-50% ammonium sulphate precipitation of total cell sonicate was analysed by HPLC on DEAE-sephadex as described (section 2.19.3). The fractions containing breakdown products of the intact A-chain were pooled and the activity was determined using the *in vitro* assay as described (section 2.20.2). The pooled fraction was analysed on Blue-sepharose CL-6B as described (section 2.19.4). The fractions containing breakdown products of A-chain were pooled and serial dilutions were analysed for activity as described (section 2.20.2).

(A) Western blot using the anti-(rabbit IgG)-alkaline-phosphatase conjugate as described (section 2.18.2). Lanes 1 and 2, recombinant abrin A-chain; lane 3, breakdown products of A-chain eluted from DEAE-sephadex; lane 4, breakdown products of A-chain eluted from Blue-sepharose CL-6B; lane 5, native abrin C.

(B) Assay for activity associated with the fraction shown in lane 3 of (A) above. Lanes 1 and 2, 0.1μg ricin A-chain minus and plus aniline treatment respectively; lane 3 and 4, 1μl of fraction shown in lane 3 of (A) above minus and plus aniline treatment respectively.

(C) Assay for activity associated with the 27kDa breakdown product of A-chain shown in lane 4 of (A) above. Lanes 1 and 2, 0.1μg ricin A-chain minus and plus aniline treatment respectively, lanes 3 and 4, 100ng, 100pg and 100fg respectively of 27kDa breakdown product of A-chain plus aniline treatment.


A)
1 2 3 4 5


B)
1 2 3 4
28S
18S
400bp
5.8S
5S

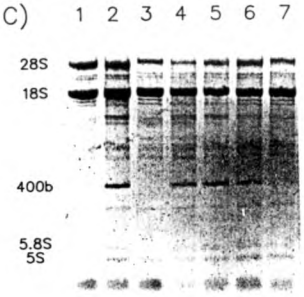

C)
1 2 3 4 5 6 7
28S
18S
400b
5.8S
5S

Figure 48B, lane 4, shows the pooled fractions containing the 27kDa protein eluted from Blue-Sepharose CL-6B analysed by Western blot. The amount of the protein was too low to detect on stained gels. The fraction in lane 4 was concentrated by lyophilisation and dilutions were analysed for activity using the aniline assay (section 2.18.2). Figure 48C shows that the 27kDa peptide retains activity and could modify rabbit reticulolysate ribosomes at concentrations as low as 10^{-10}M.

A reduction in size of 1kDa will correspond to the loss of approximately 10 amino acids. It is known that ricin A-chain retains activity when the first 9 residues at the amino terminus are deleted (May *et al.*, 1989). This may therefore suggest that in the 27kDa peptide it is the N-terminus that has been removed. A breakdown product of native abrin C A-chain of about 27kDa, which did not bind to Blue-Sepharose, was partially sequenced and the N-terminus was intact (Dr E.J. Wawrzynczak, personal communication). The activity of this peptide *in vitro* was not determined but the lack of binding to Blue-Sepharose CL-6B suggests a severe disruption in the conformation of the active site. Funatsu *et al.* (1989) showed that ricin A-chain lacking the residues from position 238 retained only 1.8% of the activity of native A-chain, and binding to cibacron blue F3GA was severely reduced. These results therefore suggest that the 27kDa peptide derived from the recombinant abrin A-chain has lost the N-terminus and the C-terminus remains essentially intact. It would be necessary to sequence the 27kDa peptide to confirm this hypothesis.

Figure 49 *Properties and activity of the 24kDa breakdown product of A-chain* The pooled fraction containing the breakdown products of the A-chain, eluted from DEAE-sephadex, were analysed on Blue-sepharose CL-6B. The void volume from the column was analysed by (A) SDS-PAGE and Coomassie blue staining, lanes 1 and 4, low range protein standards; lane 2, total cell sonicate; lane 3, void volume, and for (B) *in vitro* activity against rabbit reticulocyte lysate as described (2.20.2), lanes 1 and 2, incubation with 0.1µg ricin A-chain, minus and plus aniline treatment respectively; lanes 3 and 4, incubation with 5µl void volume, minus and plus aniline treatment respectively

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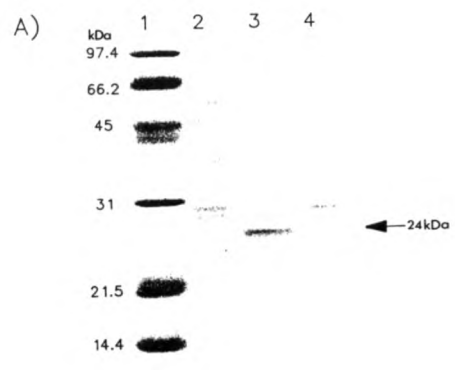

A)
kDa
1
2
3
4
97.4
66.2
45
31
21.5
14.4
24kDa

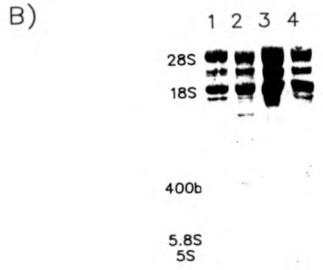

B)
1 2 3 4
28S
18S
400b
5.8S
5S

Figure 49A shows the void volume, containing the 24kDa protein, from Blue Sepharose CL-6B. An aliquot of this fraction (lane 3) was used in the aniline assay to determine if there was any activity associated with the 24kDa peptide. Figure 49B shows that no activity was detected in a sample containing an estimated 1ug of the 24kDa peptide. This suggests that loss of binding to Cibacron Blue is associated with loss of enzymatic activity. A loss of about 4kDa worth of protein sequence is considerable and at least one residue postulated to be involved in the active site is removed when 4kDa of peptide is removed from either the N-terminus, C-terminus or a combination of both. It was not unexpected that the 24kDa protein was inactive.

3.11 RECOMBINANT B-CHAIN EXPRESSION IN HETEROLOGOUS SYSTEMS

3.11.1 B-chain expression construct

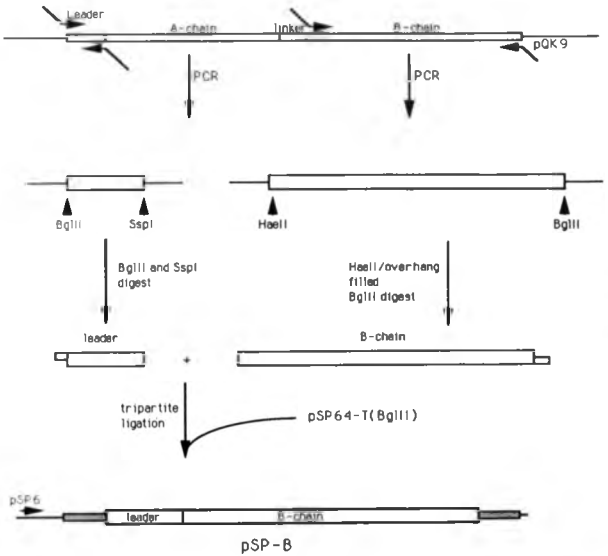
The mature B-chain sequence was recovered from pQK9 by PCR using the linker extended primers illustrated in Figure 50A. These primers when used in PCR generate the B-chain sequence on a fragment flanked by a *HaeII* site at the 5' end and a *BglII* site at the 3' end.

The preproprotein leader sequence was also isolated from the sequence of pQK9 using linker extended primers (Figure 50B). These primers, when used in PCR, generate the leader sequence on a fragment flanked by a 5' *BglII* site and by a 3' *SspI* site.

The B-chain expression plasmid designated as pSP-B was generated by a tripartite ligation containing the B-chain fragment sequentially blunted at the *HaeII* site then digested with *BglII*, the leader fragment digested with *BglII* and *SspI*, and the vector pSP64T digested with *BglII* and dephosphorylated. The cloning strategy is summarised in Figure 51.

Figure 50 *Primer design for recovery of the leader and B-chain sequences from pQK9* (A) The primers 9L-F and 9L-R and (B) 9B-F and 9B-R were used in PCR on pQK9 template to recover the leader and B-chain sequences. The corresponding coding sequence is shown above the DNA sequence, and is numbered according to Figure 27 for reference. The orientation of the primers is indicated with primers 9L-R and 9B-R representing complementary DNA. The linker regions, to generate restriction endonuclease sites are underlined

Figure 51 *Cloning strategy for the B-chain expression construct*



pSP-B

3.11.2 Mutagenesis of putative galactose-binding sites

The potential galactose binding sites

The feature of the B-chain on which research was centred was potential lactose binding ability. The majority of research to date has involved the study of the binding sites of ricin D B-chain. As discussed in section 3.7.5, the sequence of the cloned abrin B-chain has the features of the low affinity site of ricin D (1 domain) and of the 2δ domain of ricin E.

There has been relatively little work done addressing the galactose binding of the B-chain of ricin E which has the histidine residue in the galactose binding domain of the 2δ subunit. Ricin E has cytoagglutinating ability and is as toxic to mice as ricin D, however binding affinities to Sepharose 4B differ and ricin E is less toxic to cultured cells (Yamasaki *et al.* 1989b). Hatakeyama *et al.* (1989) reported that ricin E has two binding sites, a high affinity site and a low affinity site. The low affinity site was similar to that in ricin D but the high affinity site had only half the affinity for galactose as the high affinity site of ricin D. The involvement of a tryptophan residue in galactose binding at both the low and high affinity binding site was postulated from oxidation of tryptophan residues by N-bromosuccinimide in the presence and absence of lactose (Yamasaki *et al.*, 1989b and Hatakeyama *et al.*, 1990a). *N*-acetyl galactosamine did not protect the tryptophan in the low affinity site. Similar experiments using diethylpyrocarbonate to modify histidine residues in the presence and absence of lactose indicated that a histidine residue was involved in the high affinity site (Hatakeyama *et al.*,1990b) whereas modification of histidine residues had no effect on the binding at the low affinity site (Yamasaki *et al.*,1989). The histidine residue involved at the high affinity site was postulated to be either His248 or His251 and both galactopyranosides and *N*-galactosamines could protect the histidine residue from modification (Yamasaki *et al.*, 1989c). Taken together these results suggest that ricin E contains two

binding sites, one involving Trp37 is a low affinity site, the other involving His248 is a high affinity site binding both *N*-actylgalactosamines and galactopyranosides. The experimental evidence on the binding of ricin E to various sugars must be viewed with caution however. The chemical modification of a residue may alter the native conformation of the protein leading to spurious binding activity. The use of acetylated sugars to analyse binding activity of chemically modified B-chain is of limited value since the more bulky sugar may be hindered in access to the binding sites.

Yamasaki *et al.* (1989a) studied the binding of APA to specific sugars. Using fluorescence spectroscopy four tryptophan residues were determined to be involved in binding to sugars. It was assumed that APA had a single binding site per B-chain and it was concluded from the association constants for lactose that each binding site had a subsite which could bind to the saccharide linked to galactopyranoside at the non-reducing end in addition to binding to the galactopyranosyl residue. It may be possible that APA has two binding sites per B-chain which could explain the results obtained by Yamasaki *et al.* (1989a). Equilibrium dialysis was used by Ohba *et al.* (1990) to study the binding sites of abrin C B-chain. The data suggested that abrin C has two saccharide binding sites with different affinities for galactopyranosides. Spectroscopic studies indicated that the binding of galactosamines differed from the binding to galactopyranosides and suggested that a high affinity site bound to both galactopyranasides and galactosamines whereas a low affinity site bound only galactosamines. At both sites, galactosamines induced a change in the environment of tryptophan residues.

Site-directed mutagenisis of binding sites

It has previously been shown that mutation of Asn255 in ricin D B-chain to Gly abolished binding at the high affinity binding site (Wales *et al.*, 1991). Vitetta and Yen (1990) showed that mutation of Asn255 to Ala in ricin B-chain

abrogated binding by 99%. Similarly, simultaneous mutation of Lys40 and Asn46 to Gly and Met respectively abolished binding at the low affinity site (Wales *et al.*, 1991). Wales and co-workers demonstrated that loss of binding at the low affinity site abrogated binding hardly at all. Site-directed mutagenesis was carried out on the B-chain sequence of the abrin clone to determine the contribution specific residue changes made to galactose binding. The potential binding site in the 1α domain was expected to be a low affinity site and the site in the 2β domain was expected to have little or no binding activity. Rutenber and Robertus (1991) report that a histidine at position 248 in the B-chain can make hydrophobic contacts with the non-polar face of the sugar molecule but as the histidine residue is likely to be charged for most of the time this would interfere with the interaction.

The mutations carried out on the B-chain of the recombinant abrin are listed in Table 6. Mutation A1 is expected to abrogate any binding associated with the low affinity site, mutation A2 is expected to abrogate any binding associated with the high affinity site and mutation Y2 is expected to generate a high affinity binding site with similar affinity for saccharides to ricin D. The double mutant A1A2 is expected to be a non-binding mutant, and the double mutant A1A2 will allow analysis of the effect of His versus Tyr at the high affinity site independent of any binding at the low affinity site.

Table 6 *Mutations of the recombinant B-chain*

Construct	Position	Wild type	Mutant
A1	41	Lys	Ala
	47	Asn	Ala
A2	256	Asn	Ala
Y2	249	His	Tyr
A1A2	41	Lys	Ala
	47	Asn	Ala
	256	Asn	Ala
A1Y2	41	Lys	Ala
	47	Asn	Ala
	249	His	Tyr

3.11.3 *In vitro* transcription and translation

To allow comparison of the mutant B-chain with the wild type, batch solutions for the transcription and translation reactions were prepared and aliquoted out to reduce error in the final concentration of components of the systems and in pipetting.

The result from *in vitro* transcription and translation of the wild type and the five mutant B-chains is shown in Figure 52. The band of 32kDa is immunoprecipitated from the translation mix only when B-chain RNA was translated (lanes 2-7) and not in the absence of any exogenous RNA (lane 9) or on translation of TMV RNA. There is also a band of approximately 27kDa present in all lanes containing B-chain. It is unclear what this 27kDa band corresponds to.

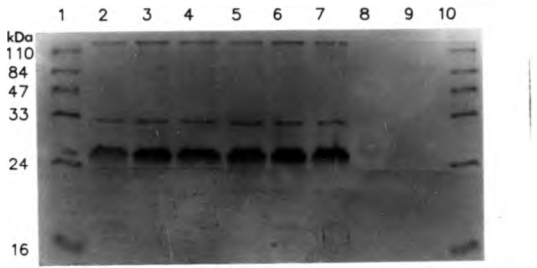
It should be noted that the amount of B-chain produced was always lower than that for ricin B-chain. Wheat germ lysate was also used for translation of the abrin-related B-chain mRNA but a product of the correct size was barely detectable. Ricin B-chain mRNA translates well in both rabbit reticulocyte and wheat germ lysate.

3.11.4 Translation of B-chain mRNA in *Xenopus oocytes*

Oocyte injections

For direct comparison of the wild type B-chain with the mutant forms, the *in vitro* transcribed mRNAs were injected into oocytes from the same ovary. The oocyte injections were performed by Professor H. Woodland (University of Warwick). Oocytes isolated from different ovaries, or from the oocytes of different animals show a wide variability in translational efficiency (personal communication, Professor H. Woodland, University of Warwick).

Figure 52 *In vitro translation of pre-B-chain mRNA* The wild type and mutant pre-B-chain constructs were transcribed *in vitro* as described (section 2.21.1). The RNA was extracted and resuspended at 1mg/ml. The transcribed RNA (1μg) was translated *in vitro* using nuclease treated rabbit reticulocyte lysate as described (section 2.20.2). The products of translation were immunoprecipitated as described (section 2.22.3) using anti-abrin sera, and analysed by SDS-PAGE and autoradiography. Lanes 1 and 10, [^{14}C]-labelled protein standards; lanes 2-8, translation of transcribed RNA of pre-B-chain, mutant A1, mutant A2, mutant Y2, mutant A1A2, mutant A1Y2 and Brome Mosaic Virus respectively and lane 9, no RNA



Expression of B-chain and solubility

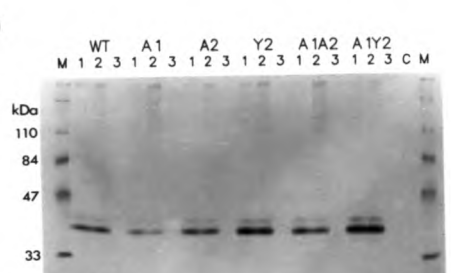
Figure 53A shows the result of immunoprecipitation from oocyte homogenates expressing the B-chain constructs. Each B-chain mRNA was successfully translated in oocytes producing immunoreactive products of approximately 38kDa. The size of the translated B-chains indicates that the proteins had been segregated to the ER and undergone core-glycosylation with removal of the signal sequence. The presence of two bands indicates differences in the degree of glycosylation. The wild type B-chain and each of the mutants were soluble and no immunoreactive B-chain was detected in the pelleted fraction.

When the oocyte homogenates were analysed 7 days after homogenisation it was found that the wild type B-chain and the mutant B-chains were insoluble. The results are shown in Figure 53B. The majority of the B-chain in each sample was found in the pelleted fraction. This is in contrast to ricin B-chain which remains essentially soluble for several weeks. Mutations in the galactose binding sites of ricin B-chain do not significantly alter the stability of this protein.

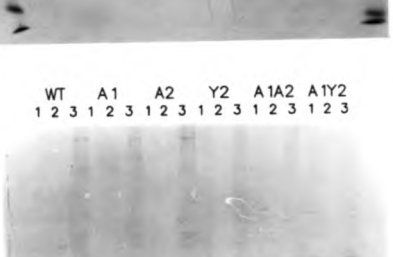
The poor stability of the recombinant abrin B-chain indicates that the protein is undergoing a conformational change on storage, leading to insolubilisation. This may be a reflection of the *in vivo* protein structure. In the plant the B-chain is associated with A-chain by a covalent bond and the B-chain may be naturally unstable once released from the A-chain. Instability may also be as a consequence of expression in the heterologous system used: disulphide bond pairings may be altered for example. The wild type B-chain sequence and the mutant sequences were initially soluble however, which indicates that at extraction the proteins are in a relatively native form and would be expected to be soluble

Figure 53 *Expression of pre-B-chain and pre-B-chain mutants in Xenopus oocytes* 100ng of RNA transcribed *in vitro* was injected into *Xenopus* oocytes. The oocytes were incubated overnight in the presence of [^{35}S]-methionine. The oocytes were homogenised and (A) product solubility was determined by centrifugation at 100,000g for 1 hour and by analysis of the pelleted and soluble fractions and (B) stability of the products was determined by analysing solubility of the proteins after 7 days storage at 4^{o}C. The samples were immunoprecipitated using anti-abrin sera and analysed by SDS-PAGE and autoradiography. Lane M, [^{14}C]-labelled protein standards; lanes 1, 2, and 3, total, soluble and insoluble fractions respectively; lane C, total fraction from mock injected oocyte

A)


WT A1 A2 Y2 A1A2 A1Y2
M 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 C M
kDa
110
84
47
33
24

B)

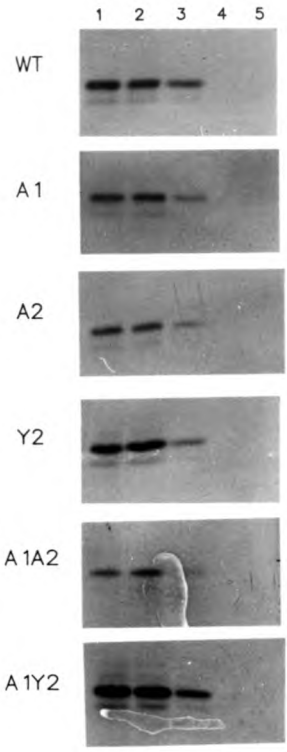

WT A1 A2 Y2 A1A2 A1Y2
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

3.11.5 Binding to immobilised lactose and asialofetuin

Binding to immobilised lactose

The ability of the abrin-related B-chain to bind to immobilised lactose was determined using the oocyte homogenates immediately after homogenisation. The results are shown in Figure 54. It is clear that both the wild type B-chain and the mutant forms do not bind to the selectin column. There was no evidence of any B-chain in the eluted fractions. The mutant Y2 may have been retarded slightly by the column since there is a very faint band present in the second wash, indicating that more than one column volume of buffer was required to remove all the B-chain. This is similar to abrin-b and abrin-c as described by Lin *et al.* (1981) which are retarded by 4B Sepharose but elute in the absence of galactose. However, ricin B-chain which has had both binding sites mutated and does not bind to saccharides exibits a similar profile on immobilised lactose. The degree of retention, if any, on the column is therefore not significant to specific binding. To check that the column was functioning, native APA was used as a control and was found to bind satisfactorily and was eluted with 50mM lactose. As an assay for saccharide binding the use of a selectin column was relatively crude. B-chain may have been binding to the column but at levels below the limit of detection of the system.

Figure 54 Analysis of binding to immobilised lactose by B-chain
A sample of oocyte homogenate was made up to 1ml with oocyte homogenate buffer. The sample was passed down a column of immobilised lactose several times to allow for binding. The column was washed three times with 1ml oocyte homogenisation buffer and the fractions were saved. Bound protein was eluted with 2ml 50mM lactose in oocyte homogenisation buffer and 1ml fractions were collected. Half of each fraction was immunoprecipitated and analysed by SDS-PAGE and autoradiography. Lane 1, starting fraction, lanes 2, unbound fraction; lanes 3 and 4, sequential wash fractions; lane 5, eluted fractions (pooled)


1 2 3 4 5
WT
A1
A2
Y2
A1A2
A1Y2

Binding to asialofetuin

The asialofetuin plate assay is both sensitive and quantitative. The wild type B-chain and the mutant B-chains were each assayed for binding to asialofetuin as described. It had been previously shown that the anti-ricin B-chain antibodies used in the assay could immunoprecipitate the abrin-related B-chain. The results of the assay are shown in Table 7. It is clear that in the presence of abrin-related B-chain the detected radiolabel was as low as the background counts indicating that if any binding had occured it was below the limit of detection. Using ricin B-chain as a positive control, this assay could detect 10ng of bound B-chain. Based on this value less than 10ng of the abrin-related B-chain was binding to the asialofetuin. The value of 10ng will be a slight over-estimation however since the antibodies used are expected to have a stronger affinity for ricin B-chain than for the abrin-related B-chain.

Table 7 *Binding of B-chain to asialofetuin* Binding to asialofetuin was determined using the asialofetuin plate assay as described (section 2.22.2). The table lists the average cpm determined from triplicate experiments

Sample	cpm	
	with asialofetuin	no asialofetuin
100ng ricin B-chain	12,932	162
50ng ricin B-chain	7,351	154
10ng ricin B-chain	1,464	136
5ng ricin B-chain	672	171
abrin B-chain	151	163
mutant A1	173	170
mutant A2	166	159
mutant Y2	179	190
mutant A1A2	154	127
mutant A1Y2	121	189
mock injected oocyte homogenate	154	169

3.11.6 Reassociation of B-chain with A-chain

Based on the sequence of the wild type B-chain the B-chain is postulated to have at least one functional binding site but of low affinity. Lack of binding of the wild type might therefore suggest that this protein is devoid of lectin activity. The mutants Y2 and A1Y2 were expected to generate a high affinity binding site however so this suggested that the protein may be incorrectly folded and the altered conformation may be leading to the increased instability of the abrin-related B-chain compared to ricin B-chain produced in oocytes.

The isolation of the B-chain away from the A-chain does not occur in nature except perhaps at the final stage of intoxication of a cell when the A-chain is translocated to the cytosol. It is possible that the B-chain remains associated with the A-chain throughout the intoxication process and is translocated with the A-chain into the cytosol. In the case of the abrin-related protein the B-chain may be stabilised by the A-chain and association between the two polypeptides is required to maintain the functional activity of the B-chain. To determine if the B-chain required A-chain for conformational activity, B-chain was mixed with the abrin-related recombinant A-chain or recombinant ricin A-chain to allow reassociation (section 2.22.3). The asialofetuin plate assay was repeated and the results are given in Table 8. There was no evidence of binding to asialofetuin when A-chain was also present in the extract.

Table 8 *Binding to asialofetuin by B-chain in the presence of A-chain* The asialofetuin plate assay was carried out as described (section 2.22.2). The table lists the average cpm determined from triplicate experiments

Sample	cpm	
	with asialofetuin	no asialofetuin
100ng ricin B-chain	13,056	157
50ng ricin B-chain	6,971	129
10ng ricin B-chain	1,576	163
5ng ricin B-chain	714	165
abrin B-chain + A-chain	134	122
mutant A1 + A-chain	111	108
mutant A2 + A-chain	117	154
mutant Y2 + A-chain	135	136
mutant A1A2 + A-chain	177	179
mutant A1Y2 + A-chain	151	162
mock injected oocyte + A-chain	134	156

It was necessary to determine if the translated B-chain did reassociate with A-chain. Following mixing of the A-chain and B-chain, samples were immunoprecipitated with anti A-chain antibodies and analysed on reducing and non-reducing gels followed by autoradiography. Only labelled B-chain which has been immunoprecipitated because of association with A-chain will produce a signal. Therefore on a non-reducing gel a protein of about 68kDa would be expected consisting of associated A-chain with B-chain, whereas on a reducing gel any A-chain-associated B-chain will appear as a band of about 38kDa. The results (not shown) indicated that there was no association of labelled B-chain with either the recombinant abrin A-chain or with ricin A-chain.

Summary of B-chain expression

Several conclusions may be drawn from the results. The wild type B-chain of the preproprotein may essentially lack any lectin activity, or the degree of binding is below the limits of detection of the assays and is therefore virtually negligable. This indicates that in the wild type B-chain both the 1α and the 2β domain binding sites are non-functional. Mutation of the 2β binding site to replace the histidine at the 2β domain with a tyrosine (mutants Y2 and A1Y2) did not generate a functional binding site as determined from passage down a column of immobilised lactose or the asialofetuin plate assay. The His to Tyr mutation was expected to generate a high affinity binding site therefore the lack of binding at the 2β domain would suggest that the conformation of the protein has been disrupted, resulting in loss of binding activity in the 2β domains of mutant Y2. This conclusion is supported by the poor stability of the B-chain compared to ricin.

3.11.7 Expression of the preproprotein in *Xenopus* oocytes

It has been shown that preproricin retains functional galactose binding domains but lacks *N*-glycosidase activity (Richardson *et al.*, 1989b). Expression of the preproabrin mRNA in oocytes would be expected to produce a more stable protein than B-chain alone: in the plant the protein is expressed as a preproprotein, the co-translational folding of the A-chain and linker sequence are likely to contribute to the subsequent folding of the B-chain, the linker is expected to stabilise the proprotein, and the B-chain remains associated with A-chain. The glycosylated linker may be important for stability or folding of the B-chain.

The primers 9L-F and 9B-R (Figure 50) were used in PCR to isolate the preproprotein sequence and this was subcloned into pSP64-T. The cloning strategy is summarised in Figure 55. The mRNA was transcribed *in vitro* as described (section 2.22.1) and translated *in vitro* in rabbit reticulocyte lysate. The result from the translation is shown in Figure 56.

The *in vitro* transcribed mRNA was injected into oocytes and the products of translation were analysed for solubility, stability and binding to immobilised lactose and asialofetuin. The results for solubility and stability are shown in Figure 57. The protein is essentially soluble immediately following oocyte homogenation (Figure 57A, lane 3). The trace of protein detected in the pelleted fraction (lane 4) is probably due to the trapping of some proprotein in the membranes, since after 14 days of storage the preprotein was found entirely in the soluble fraction (Figure 57B, lane 3).

Figure 55 *Cloning strategy to generate preproprotein expression construct pSP-PPA*

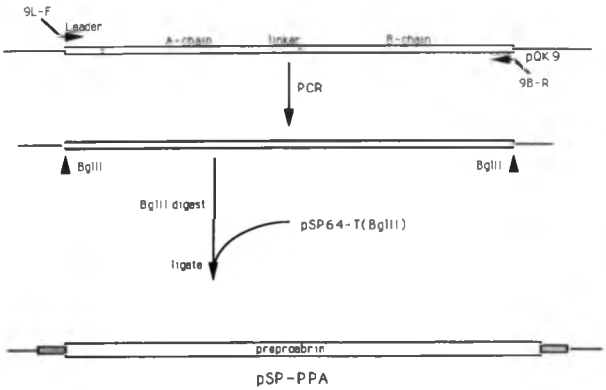


Figure 56 *Expression of preproprotein in vitro* The construct shown in Figure 55 was transcribed *in vitro* as described (section 2.21.1). The transcribed RNA was extracted and resuspended at 1mg/ml. The preproprotein mRNA was translated using nuclease treated rabbit reticulocyte lysate as described (section 2.21.2) in the presence of [^{35}S]-methionine. The products of translation were immunoprecipitated with anti-abrin sera and analysed by SDS-PAGE and autoradiography. Lane 1, translation without exogenous RNA; lane 2, translation of Brome Mosaic Virus RNA; lane 3, translation of preproabrin RNA; lane 4, [^{14}C]-labelled protein standards

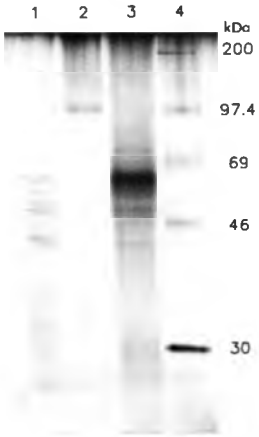
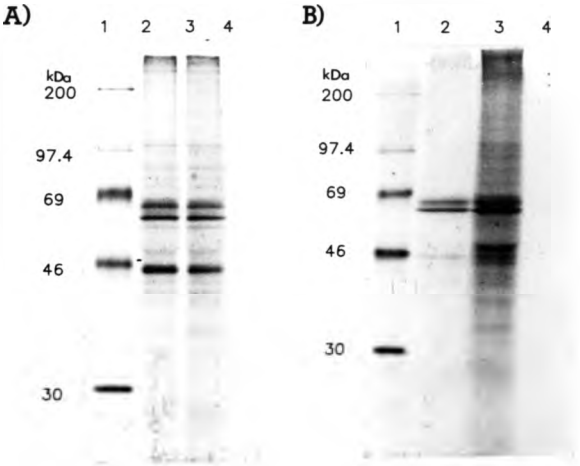


Figure 57 *Expression of the proprotein in Xenopus oocytes* RNA encoding the preproprotein was injected into *Xenopus* oocytes as described (section 2.22.1) and the oocytes were incubated overnight in the presence of [^{35}S]-methionine. The oocytes were homogenised and the insoluble protein fraction was recovered by centrifugation at 100,000g for 1 hour. The homogenates were stored for 14 days at 4^{o}C and analysis of solubility was repeated. The fractions were immunoprecipitated and analysed by SDS-PAGE and autoradiography. (A) Solubility at extraction and (B) solubility after 14 days of storage. Lane 1, [^{14}C]-labelled protein standards; lane 2, total homogenate; lane 3, soluble fraction; lane 4, insoluble fraction



The increase in stability of the proprotein compared to the B-chain expressed in oocytes indicates that the A-chain and linker are contributing to the conformational stability of the B-chain. The glycosylation of the linker may be important for correct folding. The amount of abrin-related proprotein produced is significantly lower than that obtained from translation of preproricin. Of a variety of mRNAs translated in oocytes, preproricin or pre-ricin B-chain mRNAs are translated at some of the highest levels, suggesting that oocytes contain all the components involved for efficient translation of ricin-derived mRNAs. It is probable that other mRNA species, which include the abrin-related mRNAs described here, are not optimally transcribed due to the lack of certain components of the protein synthesis machinery. Recently the role of chaperones has become increasingly evident (Ellis, 1990). It can readily be appreciated that the efficiency of folding of heterologous proteins into the native form in oocytes will depend on the presence of components which can substitute for those found in the homologous system.

The binding of the proprotein form of the abrin-related protein to immobilised lactose was determined and the results are shown in Figure 58. The proprotein was eluted from the column in the absence of lactose indicating that the protien was devoid of lactin activity.

The asialofetuin plate assay was carried out and the results shown in Table 9 confirm that the proprotein had no lectin activity. Again, the limit of detection for ricin B-chain was in the range of 10ng.

Figure 58 *Binding of proabrin to immobilised lactose* A sample of oocyte homogenate was made up to 1ml with oocyte homogenate buffer. The sample was passed down a column of immobilised lactose several times to allow for binding. The column was washed three times with oocyte homogenisation buffer and the fractions were saved. Bound protein was eluted with 2ml 50mM lactose in oocyte homogenisation buffer and 1ml fractions were collected. Half of each fraction was immunoprecipitated and analysed by SDS-PAGE and autoradiography. Lane 1, [^{14}C]-labelled protein standards; lane 2, starting fraction; lanes 3-5, sequential wash fractions; lanes 6 and 7, sequential eluted fractions

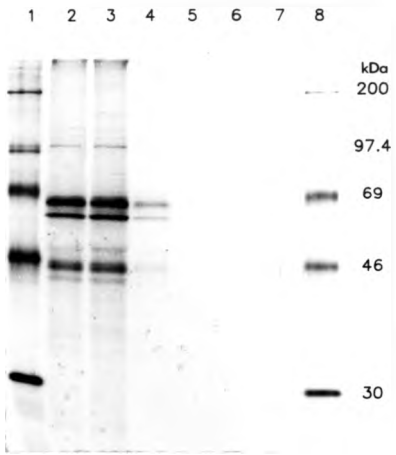


Table 9 *Binding of proabrin to asialofetuin* The asialofetuin plate assay was carried out as described (section 2.22.2). The average cpm determined from triplicate experiments are shown

Sample	cpm	
	with asialofetuin	no asialofetuin
100ng ricin B-chain	13,816	174
50ng ricin B-chain	8,084	182
10ng ricin B-chain	1,690	201
5ng ricin B-chain	707	170
proprotein	226	195
mock injected oocyte homogenate	157	172

3.11.8 Summary

The close similarity between ricin and the abrin-related clone suggested that the properties of the two proteins would be similar and that the systems used for analysis of ricin B-chain would be applicable. Based on the knowledge about ricin B-chain and the results obtained from experiments on the galactose binding sites of ricin B-chain, a hypothesis for binding of the abrin-related B-chain was made: the abrin clone contains a low affinity site in the 1α domain of the B-chain and the putative binding site in the 2β domain is disrupted by the histidine residue.

The results obtained indicate that the B-chain of the abrin-related protein lacks specific lectin activity. However the lack of binding of the mutant B-chains Y2 and A1Y2 indicate that there is a significant difference between ricin B-chain and the B-chain studied here. The poor stability of the expressed B-chains is indicative of conformational change and this conclusion is supported by lack of reassociation between the B-chain and exogenously added recombinant A-chain. The abrin B-chain may be more stable when expressed in other heterologous systems. Ricin B-chain has been expressed in yeast (Richardson *et al.*, 1988b) and glycosylated functionally active ricin B-chain has been expressed in monkey kidney cells (Chang *et al.*, 1987).

In an attempt to overcome the problem of conformational stability, the preproprotein sequence was expressed in oocytes. The preproprotein form is expected to retain the activity associated with the B-chain, but lacks the activity of the A-chain. The improved sability of the abrin-related proprotein over the B-chain supports the hypothesis that the abrin-related protein is dependent on quaternary structure for solubility and therefore activity. The lack of binding activity of the proprotein indicates that the cloned abrin-related gene does not encode a lectin despite retention of the key residues involved in tertiary structure and hydrogen bonding.

The 1 domain is non-functional therefore there must be structural changes in this domain. There are certain changes around a crucial Trp residue compared to ricin, shown below:

the abrin B-chain	N R I I A W K C
ricin B-chain	N A I Q L W P C

The Arg to Ala, the Ile to Gln and the Lys to Pro changes are chemically pronounced and as these changes concentrate in the putative binding site and interact with each other, the binding site may be seriously disturbed despite retention of the key hydrogen-bonding residues.

The lack of binding at the 2δ domain supports the conclusion of Rutenber and Robertus (1991) that the charge on the histidine residue disrupts the hydrophobic interaction with a saccharide. There may be other less obvious changes in the 2δ domain which may alter structure and render the 2δ domain non-functional. It would be necessary to mutate the histidine to a tyrosine and express the B-chain in the preproprotein form to confirm if a histidine residue alone is responsible for non-functionality.

Lack of lectin activity is supported by the increased internal divergence of the abrin B-chain compared to ricin (refer to Figure 26B). The constraints of natural selection will be lessened and the abrin B-chain may therefore have accumulated amino acid substitutions at a faster rate than ricin B-chain.

The abrin-related protein is devoid of lectin activity and therefore is not expected to be a cytotoxin. The B-chain is responsible for cell entry and it is now evident that the B-chain plays an important role in the intracellular trafficking of the A-chain.

Binding to the cell surface has been extensively investigated. As discussed in the introduction, the bound holotoxin is endocytosed through clathrin coated pits. The non-clathrin coated pits are almost certainly another endocytic

route for ricin and Sandvig and van Deurs (1990) have clearly demonstrated the uptake of ricin when the clathrin coated pit pathway is blocked. Anti-tumour activity of ricin, against a neoplastic hepatic cell line, was shown to be related to the binding of the B-chain to high affinity receptors which led to endocytosis via neutral vesicles as opposed to acidic vesicles (Decastel *et al.*, 1989). A holotoxin which lacks lectin activity cannot gain entry to cells by the usual endocytic pathways. The abrin clone is glycosylated in both the A-chain and the B-chain and it is highly likely that the oligosaccharide side chains will contain mannose. The holotoxin would be recognised by the mannose receptors on reticuloendothelial cells and therefore could gain entry to certain cells.

Bellilli *et al.* (1990) have used ricin labelled with fluorescein to follow the internal pathway of ricin in living cells and ricin was shown to reach late endosomal vesicles distinct from the lysosomal vesicles. Hansen *et al.* (1989) have used immunogold cytochemistry to demonstrate the colocalisation of internalised ricin with the high-molecular weight membrane glycoprotein MAM-6 of human breast epithelial cells in the trans-Golgi network. The Golgi-network has been shown to be important for ricin toxicity in Golgi-defective mutants of chinese hamster ovary cells which exhibit increased sensitivity to ricin. The increased cytotoxicity of ricin was not due to different mechanisms of processing of the ricin in the mutant cells.

Recently, experiments using brefeldin A have confirmed that ricin must reach the trans-Golgi network for cytotoxicity (Yoshida *et al.*, 1991). Brefeldin A is a fungal metabolite which dramatically alters the structure of the Golgi-network causing the Golgi contents and membranes to distribute to the ER. The Golgi stacks disassemble but markers for the trans-Golgi network are unaffected (Lippincott-Schwartz *et al.*, 1990). Brefeldin A blocks the transport of glycoproteins from the ER to the Golgi (Takatsuku and Tamara, 1985) and blocks the

transport of VSV protein G to the plasma membrane (Vance *et al.*, 1991). Brefeldin A protects cells from the action of ricin and abolishes the enhancement of toxicity seen by tunicamycin and ammonium chloride. It is postulated that ricin must move through the trans-Golgi network into cis/medial and/or trans-Golgi regions before release into the cytosol suggesting that ricin requires uninterrupted membrane transport between endosomes and the Golgi-complex for cytotoxicity.

Manske *et al.* (1989) studied the trafficking of an anti-CD5 antibody linked to ricin A-chain or to intact ricin. The A-chain-containing immunotoxin was less toxic than the ricin-containing immunotoxin. It was determined that the B-chain slowed trafficking to lysosomes and that the rate-limiting step in the differences in toxicity was due to the rate of transfer to the lysosomes. A bispecific antibody recognising ricin A-chain and a carcinoembryonic antigen was potentiated by ricin B-chain in the presence of galactose indicating an intracellular role for the B-chain.

It is possible that there may be modification or processing of the toxin by enzymes in the trans-Golgi network. The B-chain may therefore determine the intracellular routing of the A-chain to a translocation-competent compartment and be involved in the translocation step itself. The abrin described here may function as a component in the translocation process in cells to which it has gained entry through mannose recognition. Most recently however, ricin lacking galactose-binding activity has been shown to be non-cytotoxic to macrophages (personal communication, Dr R. Youle, National Institutes of Health, Bethesda, Maryland, USA). The B-chain cannot bind to cell-surface receptors and is recognised by mannose receptors. Wawrzynczak *et al.* (1991) have shown that blocked ricin immunotoxins were more efficient than ricin A-chain containing immunotoxins potentiated with B-chain. It was postulated that the blocked ricin becomes unblocked after binding to the target antigen, the galactose binding sites are exposed and contribute to cell killing. These results indicate

that the galactose binding activity associated with the B-chain plays an intracellular role in addition to cell surface binding. The B-chain may recognise glycoproteins or glycolipids intracellularly and thereby determine trafficking of the A-chain or translocation. It would be of interest to determine the cytotoxicity of the abrin-related protein in this system. Lack of cytotoxicity will confirm the results of Youle. If the abrin-related protein was toxic to intact macrophages however this would indicate a role for the B-chain independent of galactose binding.

The presence of a non-lectin toxin in seeds is therefore unexpected. There are no other detailed reports of a non-lectin type II ribosome-inactivating protein. The isolation method used by most laboratories of passage down Sepharose-4B column depends upon lectin activity therefore it is possible that the non-lectin forms have not been detected as they pass directly through the column. Roy *et al.* (1976) described an *Abrus* lectin which had no cytohaemagglutinating activity and did not bind galactose.

The clone isolated may not be translated in the plant. From the nucleotide sequence of the untranslated regions, the gene has all the expected features of a transcribed gene and the long open reading frame which shows extensive homology to the other type II ribosome-inactivating proteins suggests that this is not the case.

There is clearly some evolutionary selection to maintain galactose binding domains in the ricin toxins. Ricin retains two binding sites of potentially six ancestral galactose binding domains, RCA has retained perhaps only one functional binding site per B-chain. It is possible that the abrin-related gene has lost all six.

The function of the type II ribosome-inactivating proteins in nature remains speculative. They are clearly storage proteins which are hydrolysed to provide a source of amino acids in the germinating seed. Evolutionary selection for galactose binding cannot be related to a role in protein

storage. The interchain disulphide bond must be broken for A-chain activity therefore after removal of the linker region the presence of the B-chain maintains the protein in an inactive form. This may be sufficient to maintain selection for the B-chain in evolutionary terms, independent of galactose binding.

4. SUMMARY AND OVERALL CONCLUSIONS

An abrin-related sequence was isolated which encodes a preproprotein. Analysis of the sequence revealed that the leader peptide encodes a 19 amino acid N-terminal signal sequence. The A-chain sequence is homologous at the amino acid level with abrin C A-chain and ricin A-chain. The residues known to be important for *N*-glycosidase activity of ricin A-chain, determined from the 3-dimensional structure and from mutational analysis, are all retained in the A-chain sequence reported here. The B-chain sequence is 60% similar to ricin B-chain at the amino acid level and exhibits the same pattern of subdomain duplication postulated for ricin B-chain i.e. $[\lambda(\alpha\beta\gamma)]_2$. The residues which are critical for folding of the subdomains and for the interactions between the subdomains of the B-chain are retained.

The A-chain of the abrin-related preproprotein was expressed in *E. coli*. The yield of soluble A-chain was increased to greater than 6% of the total protein by cytoplasmic expression of the A-chain sequence which had been maximised for A+T content at the 5' end of the coding sequence and by growth of the host *E. coli* at 30°C. The recombinant A-chain was purified to greater than 99% purity from crude cell sonicates. The purified A-chain is at least as active as ricin A-chain against rabbit reticulocyte lysate and yeast ribosomes. Chloroplast and *E. coli* ribosomes were shown to be resistant to the abrin A-chain.

The B-chain of the preproprotein has been expressed in *Xenopus* oocytes. The isolated B-chain is unstable and devoid of lectin activity. Lack of lectin activity was confirmed by expression of the preproprotein sequence in *Xenopus* oocytes which lacked ability to bind lactose or asialofetuin. It is surmised from the results that the environment of the tryptophan residue in the 1α binding domain is crucial for function and that a histidine in the 2β domain prevents binding to saccharides. Lack of lectin activity of the abrin-related

protein is supported by the increase in the internal divergence of the subdomains compared to ricin B-chain.

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